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The Activities of the Tube Feet of *Asterias rubens* L.

I. The Mechanics of Movement and of Posture

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With seven Text-figures

CONTENTS

I. INTRODUCTION

*A*STERIAS RUBENS, under the normal circumstances of its life in tide pools and in the deeper waters of the continental shelf, displays a range of activities which include locomotion, the capture of food, and the maintenance of a posture in which the oral surface is kept in apposition to the substratum. These and other organismal activities require an harmonious integration of movement of the different parts and organs of the body, chief among which are the tube feet and the muscular walls of the arms. These integrated movements of widely separated systems contrast vividly with the strictly autonomous behaviour of the spines and pedicellariae situated on various parts of the lateral and dorsal surfaces of the arms and disk.

It is apparent from the work of Romanes and Ewart (1881), Preyer (1886), Demoor and Chapeaux (1891), Jennings (1907), Mangold (1908), and Dieb-schlag (1938), among other authorities, that the ability of the tube feet and myodermal wall of the arms to take part in organismal responses is dependent on an innervation which includes nervous connexion with the neurones and tracts of the radial nerve cord and circumoral nerve ring, and that the autono-mous behaviour of the spines and pedicellariae is a consequence of their being innervated solely through the peripheral plexus of the dorsal sheath (i.e. the integument lateral to the tube feet). Some of the implications of peripheral and central innervation in terms of the integration of activity in starfishes have been discussed in a recent review (Smith, 1945). The present paper, on the kinematics of tube foot activity, is intended to serve as an introduction to a

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more detailed examination of the mechanisms underlying nervous integration in the starfish as evidenced by an analysis of the circumstances of stimulation and innervation appropriate to the appearance of the reflexly and centrally controlled activities of the feet.

II. The Movements and Postures of the Feet

The activities of a starfish podium comprise a number of readily recognizable movements and postures all of which originate in the contraction of smooth muscle fibres contained within the wall of the foot and of its annectant ampulla. Text-fig. 1, which illustrates schematically the arrangement and
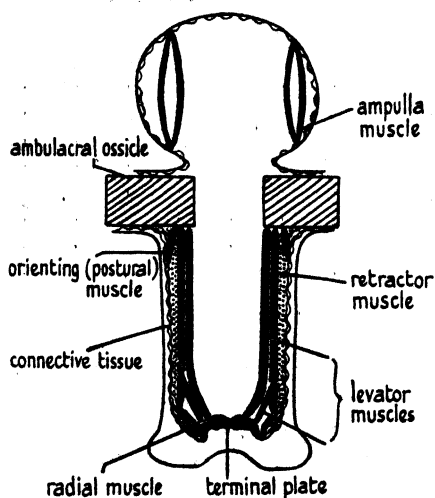
Text-fig. 1. Diagram of a longitudinal section through a foot and ampulla of *A. rubens* showing the arrangement of the principal muscle systems.

distribution of the principal systems of fibres, shows two series of muscles, the vertically running circumferential fibres of the ampulla and the longitudinal retractor muscles of the column of the podium, which have long been recognized (Greeff, 1871). The figure includes, in addition, three muscle systems not previously figured, namely, the basal sheath of orienting fibres, the radial fibres of the terminal plate, and the levator fibres of the plate, while omitting, for reasons later to be given, the musculature of the sucker figured and described by Cuénot (1891) and Chadwick (1923).

The muscles are separated from the fluid-filled cavity of the foot and ampulla by a thin coelomic epithelium and are bounded externally by a layer of connective tissue, to the collagen fibres of which they are attached either along their entire length or at their extremities. When the muscles contract, they initiate movements of the foot either by pulling directly on its wall or by exerting pressure on the fluid contained within the virtually closed system of

the foot and ampulla. It will be the purpose of this account to define the particular muscle systems responsible for each activity and to examine the part played by antagonistic muscles, the hydrostatic skeleton, and the connective tissue sheath in the shaping of each movement and posture of the foot.

III. Protraction and Retraction of the Foot

Only brief reference will be made to these movements as their kinematics have been discussed in some detail in a previous paper (Smith, 1946).
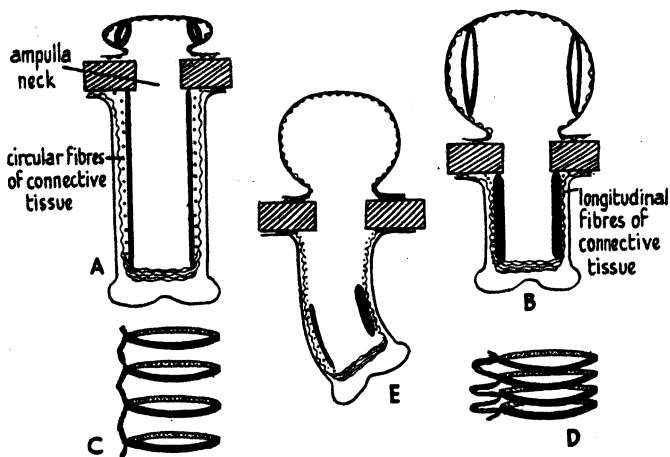
TEXT-FIG. 2. A, B, and E. Diagrams showing the conditions of contraction and relaxation of the ampulla muscles and the retractor fibres of the foot during (A) protraction, (B) retraction, and (E) localized bending of the podium. Muscles in contraction are represented by the thicker, and relaxed muscles by the thinner, of the black bands. C and D show how the longitudinal and circular fibres of the connective tissue sheath within the column of the foot are arranged when the foot is (C) protracted, and (D) retracted.

Protraction (Text-fig. 2A) is initiated by the contraction of the ampulla muscles whereby fluid is expelled through the neck of the ampulla into the cavity of the podium. The inner layer of the connective tissue sheath of the foot comprises circular fibres (Text-fig. 2A), which, being inextensible, resist the pressures exerted on the side walls of the foot by the contained fluid and prevent a lateral bulging of the column; the effective pressure of the fluid is exerted against the terminal plate and the foot protracts. During protraction the longitudinal fibres of the outer layer of the collagen sheath, which in sections of a retracted podium (Text-fig. 2D) are seen to be much convoluted, gradually straighten; though at the normal limit of protraction (Text-fig. 2C), determined in the first place by the fluid content of the ampulla, they are still somewhat folded. By pulling at the foot it is possible to extend it farther, but attempts to stretch it beyond about twice its normal protracted length invariably result in the rupture of the wall of the column. In view of the observed degree of folding of the longitudinal fibres at various extensions of the foot it

seems reasonable to infer that amputation occurs at the point when the longitudinal fibres are fully tautened.

Withdrawal of the foot is brought about by the contraction of the muscles of the retractor sheath, fluid being expelled from the diminishing cavity of the foot into the ampulla, the muscles of which relax. So long as the conditions of retraction are comparable around the entire periphery of the sheath the foot retains its cylindrical form (Text-fig. 2B), but if there is differential contraction of the individual fibres, the foot bends towards the more contracted side (Text-fig. 2E). Localized bending movements of this kind are invariably exhibited by feet during their withdrawal from the substratum at the end of the backward step (Text-fig. 7, A4). In this instance the curvature arises as a result of the difference in length of the posterior and anterior faces of the podium (relative to the direction of the step): the former, being under greater tension at the moment of release, becomes concavely curved. As will later be shown, localized bending movements may also be evoked by unilateral stimulation of the foot column.

IV. Attachment and Detachment of the Sucker

Mucus undoubtedly plays an important part in the adhesion of starfish podia to solid surfaces, but the principal adhesive force in feet possessing a terminal sucker is provided by suction (Paine, 1926). Niemiec (1885) and Preyer (1886) attributed the suction effect to the cupping of the centre of the disk with a consequent lowering of the pressure within the fluid of the suction cavity. Whereas there has been general agreement on this point there is less unanimity concerning the disposition and role of the muscle systems by which the suction mechanism is operated.

In 1891 Cuénot described and figured a system of radially arranged muscle-fibres within the sucker of the foot of *A. rubens*, distal to the terminal plate, and the view has been expressed (Sedgwick, 1927) that these muscles, by their contraction, cup the disk and fix the sucker. Notwithstanding Chadwick's (1923) confirmation of Cuénot's account, it is certain, however, that these radial fibres are not muscle but connective tissue. In sections stained with Heidenhain's iron-alum haematoxylin or with Delafield's haematoxylin and eosin, they may easily be mistaken for muscle, but when care is taken to differentiate myosin from collagen by the use of polychrome dyes such as Mallory's triple stain it is evident that muscle-fibres are absent from the disk and that the suction mechanism is operated by muscles wholly extrinsic to the sucker.

Text-figs. 3A and 3B show the principal systems of muscle-fibres and the disposition of the collagen connective tissue as seen in a median sagittal section of an *Asterias* foot, the material used being fixed in Heidenhain's 'Susa' mixture and stained in Mallory's triple stain. Although many of the fibres of the retractor musculature have a distal termination on the side wall of the podium, some of them (the levator muscles of the diaphragm) converge towards and are attached to the central area of the terminal plate of connective

tissue. The upper and lower limits of the plate are indicated in the figure by the broken white lines and it will be seen that the centre of the plate (the diaphragm) is very much thinner than is its margin. The latter forms a thickened rim continuous, proximally, with the cylindrical connective tissue sheath investing the cavity of the foot. Distally, it is drawn out into a series of vertical laminae which thrust into the substance of the sucker as a series of radially arranged wedges, the arrangement of which is well seen in transverse sections through the sucker (Text-fig. 5A). Along a considerable part of the
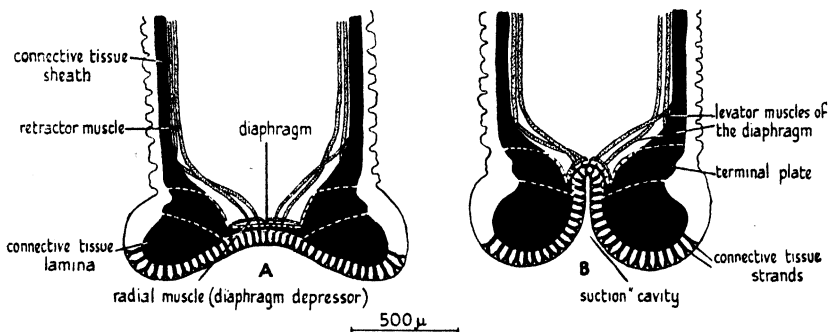
TEXT-FIG. 3. Diagrams of median sagittal sections through the distal end of (A) a protracted, and (B) a retracted, unattached, podium of *A. rubens*. The outlines of the foot and the distribution of the connective tissue (indicated by the black shaded areas) are, in each instance, accurately represented. Muscles are figured schematically.

free margin of each lamina, and over the distal surface of the centre of the terminal plate, the connective tissue is frayed out into numerous strands (Text-fig. 3B), the individual fibres of which interdigitate with, and are attached to, the ectodermal cells of the sucker. When the muscles which are inserted on the upper surface of the diaphragm contract they cause it to be invaginated into the hydrocoel. The doming of the plate is clearly seen in sections through fully retracted, but unattached, feet (Text-fig. 3B). In this instance the tensions developed in the contracting musculature and transmitted through the collagen fibres of the sucker have caused a slight inward and upward rotation of the two laminae and a perceptible reduction in the diameter of the suction disk. A drawing-in of the disk is not, however, to be observed in feet the suckers of which are in contact with the substratum at the time of contraction of the retractor musculature.

Under these latter conditions (Text-fig. 4A) the levator fibres of the diaphragm exert a tension with a component (N) normal to its surface. The consequent tendency for the volume of the suction cavity to increase results in the pressure (p) within its contained fluid falling below the level of the pressure (P) acting on the outer wall of the foot. Suction will occur provided that the lateral wall of the cavity resists the tendency to collapse under the reduced pressure and provided also that the attached margin of the sucker does not

slip inwards. It is, of course, evident, in view of the fact that suction does occur when the levator fibres pull on the terminal plate, that there is neither slipping of the foot nor a collapse of its wall, but the interest of the mechanism lies in the manner in which the properties and arrangement of the muscles and connective tissue ensure the rigidity of the wall during suction.
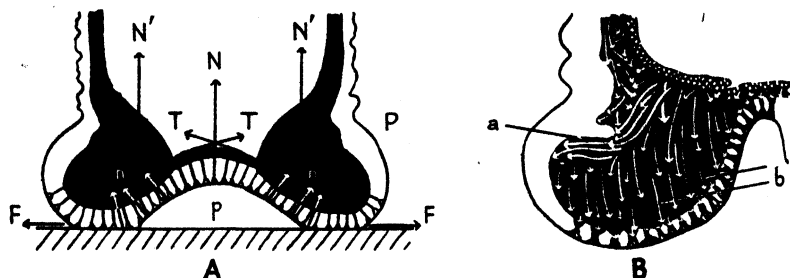
TEXT-FIG. 4. A. Diagram of a median sagittal section through the distal end of a tube foot of *A. rubens* showing the distribution and direction of the forces acting on the wall of the suction cavity during the attachment and detachment of the sucker. (*p*) is the pressure within the fluid of the suction cavity, (*P*) the pressure outside the foot. B. A part of the same section showing the orientation of the collagen fibres within the terminal plate and one of the radial laminae of the sucker. In both figures the connective tissue is shown in black. (*a*) the horizontally oriented fibres of the outer margin of the lamina; (*b*) the longitudinally oriented fibres terminating on the distal and inner edges of the lamina.

The tendency of the margin of the sucker to slip inwards is resisted by centrifugally acting frictional forces (*F*) and by the horizontally and centrifugally acting components of the forces (*n*). These latter forces are the tensions developed in the connective tissue laminae of the sucker as a result of the contraction of the retractor muscles *N' N'* inserted around the periphery of the terminal plate. The terminal plate and the laminae are composed of a network of collagen fibres which have the property of being inextensible when subjected to forces applied along their length. While (Text-fig. 4B) the proximal face of the terminal plate is made up of closely matted circumferential fibres—in effect the most distal of the circular fibres of the inner zone of the cylindrical sheath of the column—the wedge-shaped laminae are composed of fibres which have a predominantly longitudinal orientation. As will be seen from Text-fig. 4B, some of the fibres (*a*) curve outwards to form the thickened margin of the upper and outer surface of the lamina, but for the most part (*b*) they run downwards while turning all the while towards the midline of the foot. The result of this arrangement is that by far the greater number of the fibres converge on the distal and inner margins of the lamina, to be inserted at right angles to its surface. It is along a length of the lamina exactly corresponding to the line of normal insertion of the main series of fibres that the collagen tissue is frayed out into the branching strands which ultimately terminate between the cells comprising the wall of the adhesive disk and suction cavity. The inextensibility of the collagen fibres, their orientation normal to the wall of the cavity and disk, and the arborescent

endings of the system collectively contribute to a mechanism that is admirably adapted for translating, with maximum effect, the tensions (N') developed in the contracting muscles of the retractor sheath into forces (n) acting outwards from all parts of the lateral wall of the suction cavity. The rigidity of the wall of the cavity being thereby assured, the suction mechanism can operate on the principle of a piston (the central diaphragm of the terminal plate) tending to be drawn up within a rigid cylinder (the margin of the sucker).
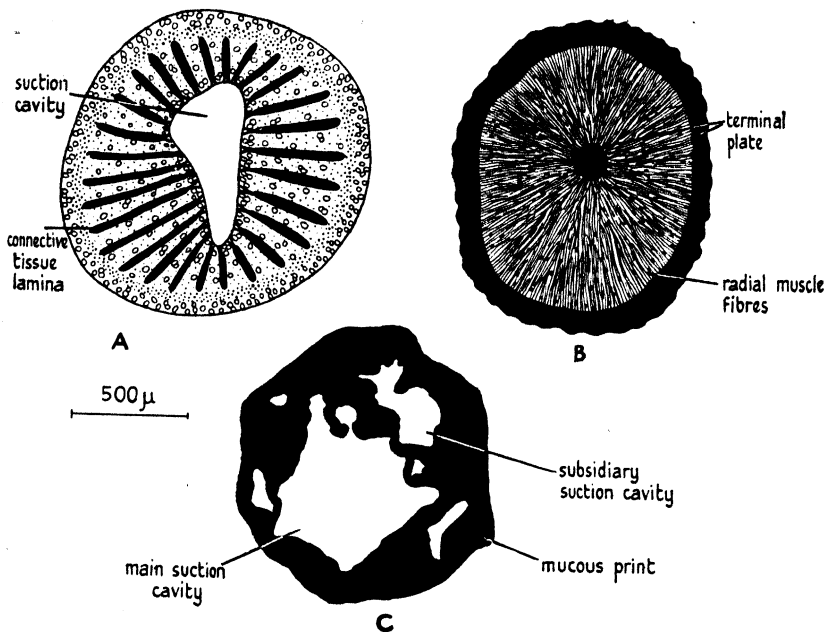
TEXT-FIG. 5. A and B. The structures seen in transverse section of the distal end of a foot of *A. rubens*. A. Below (distal to) the terminal plate. B. Through the upper (proximal) surface of the terminal plate. C is a drawing of the mucous print of an attached sucker. The area of contact of the sucker with the substratum is shown in black.

Mucous prints left by the suction disk (Text-fig. 5C) show that, in addition to the main suction cavity, there are a number of subsidiary spaces functioning as secondary centres of negative pressure. As will later be shown (p. 8), they may in addition play an important part in effecting the detachment of the disk.

The primary condition of detachment of the sucker is that the forces tending to hold it to the substratum shall be opposed by greater forces acting in the opposite direction. Forces tending to pull the foot away from the substratum may be generated within the foot itself or be applied externally by the traction of the animal as a whole as, for example, during locomotion. As we have seen, however, the negative pressure of suction will hold against these traction

forces beyond the point of rupture of the column of the foot and of amputation of the podium. Before a foot can be withdrawn undamaged from the substratum its disk must first cease to act as a sucker.

Absence of suction implies that the pressure within the fluid of the suction cavity has become equilibrated with the pressure acting on the outer wall of the disk. There appear to be two possible ways of effecting this change: (1) by allowing the outside sea-water to communicate with the fluid of the suction cavity, and (2) by the exertion of a pressure on the walls of the suction cavity equal to the negative pressure of suction.

It will be seen on examining a sucking disk prior to and at the moment of withdrawal of the foot from the substratum that, on occasions, the margin of attachment exhibits a slight wrinkling. One effect of this wrinkling will be to enlarge some of the subsidiary cavities of the suction disk and to permit of their communication one with another. If the water channels so formed lengthen sufficiently in a radial direction they will ultimately open both to the outside of the disk and into the central cavity and the disk will no longer be able to adhere by suction. The frequent detachment of the foot immediately after wrinkling has occurred appears to offer direct evidence of the actual operation of this mechanism.

It is, however, more usual for sucking disks to become detached without the occurrence of any previous wrinkling. In this event the walls of the suction cavity have presumably been subjected to forces tending to press them inwards and so to increase the pressure within the contained fluid. As will be seen by reference to Text-figs. 3 and 4A, there are no mechanisms by which an inwardly acting pressure can be exerted on the lateral walls of the cavity. These walls, solidly built and firmly attached at their base to the substratum, will not, moreover, yield to centrifugally acting forces such as will be developed when the pressure within the suction cavity rises. In the detachment, as in the attachment of the sucker, they therefore remain relatively immovable and rigid.

The central diaphragm of the terminal plate, forming the roof of the cavity, is, however, in different case. A thin circular disk with a slight convexity into the hydrocoel, it has attached to its upper surface a number of radially arranged muscle-fibres (Text-figs. 3A, 5B, radial muscle) which, originating on the inner margin of the thickened rim of the terminal plate, are inserted at the centre of the diaphragm. It will be evident that the whole relations of these radial muscles, their limitation to the one part of the wall of the suction cavity that is capable of being distorted, their radial arrangement, and their origin on a relatively immovable plate, are such as to ensure that, on contracting, they will exert forces acting tangentially to the surface of the diaphragm (Text-fig. 4A (*T*)) and so, by tending to depress the diaphragm, will cause a rise in pressure within the fluid of the suction cavity and the eventual cessation of suction.

The two methods of release from suction, by a wrinkling of the sucker and by the exertion of tangentially acting forces on the surface of the central

diaphragm of the terminal plate, are both brought about by the contraction of the radial muscles. Attachment of the sucker results from the contraction of the longitudinally oriented levator fibres of the diaphragm. Consequently these two systems of muscles are functionally antagonistic. Since, however, they may at times be in simultaneous contraction, as, for example, at the moment the sucker is pulled away from the substratum, they cannot be regarded as standing solely in the relation of reciprocally contracting and relaxing systems. It is interesting in this connexion to note, as will be shown in a later communication, that they are innervated through different systems of nerve arcs. There is accordingly no *a priori* reason why they should always act reciprocally.

V. Postural Pointing

These activities include all postures of the feet that can be said to be oriented in the sense that the podia, instead of hanging vertically downwards from the under surface of the arm, have their long axes directed obliquely to the surface. Postural pointing is exhibited during the various phases of the ambulatory step (Text-fig. 7B, 1–4), and in lateral pointing, in which latter movement the foot swings directly outwards from the mid-line of the arm.

Hamilton (1922) alone of previous authors has considered the implications of these postures in terms of the muscular anatomy of the foot. He found that the distal part of the column of a starfish foot can be twisted without affecting the angle of orientation of the podium, and concluded from this that the orienting musculature must lie towards the base of the foot. One doubts whether it is possible to draw this conclusion from a repetition of Hamilton's experiment on the foot of *A. rubens*. The podium, if allowed to attach to a needle, can be twisted readily enough but its orientation, while so attached, is too much influenced by external forces for any assessment to be made of the freely developed angle of orientation. Moreover, as soon as the needle is removed, the foot withdraws and its former pointing attitude is lost.

The occurrence of a basal orienting musculature can, however, be justified on other grounds, in spite of the fact that it is not to be distinguished in sections of the foot as an anatomically distinct system of fibres. When longitudinal sections through a foot, killed and fixed in the pointing position, are examined (Text-fig. 6) it is seen that the side of the podium which makes an acute angle with the under surface of the arm is not curved but is wrinkled at its base. These circumstances suggest that pointing results from the unilateral contraction either of short fibres within the base of the foot or of the most proximal parts of the long fibres of the retractor sheath. The latter hypothesis leads to evident difficulties. It necessitates, for example, the supposition that, during the change from the retracted (Text-fig. 7, B1) to the protracted pointing position (B2), the basal part of each of the protagonistic fibres remains contracted at a constant length while its more distal portion undergoes extension. Or again, it requires that, during the backswing of the foot (Text-fig. 7, B2 to B3), the proximal parts of the protagonistic and

antagonistic fibres shall undergo reciprocal contraction and relaxation while maintaining a substantially unaltered length distally. While such sequences and combinations of accurately graded differential contractions within the limits of a single fibre, applying *mutatis mutandis* to all the fibres of the ring, are not beyond the bounds of possibility, it seems more reasonable to suppose, in view of all the circumstances, that the postural and retractor muscles not only function as, but are indeed, in actual fact, morphologically separate systems.
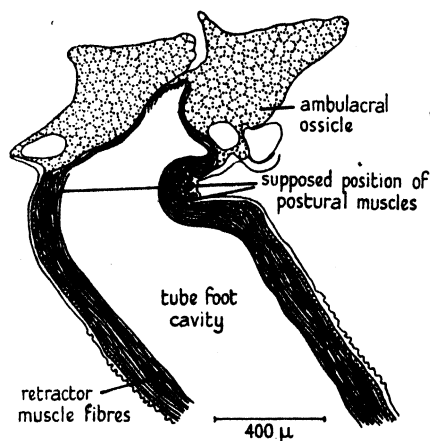
TEXT-FIG. 6. A longitudinal section through the base of a pointing foot of *A. rubens.*

It must further be supposed, since the foot can be directed towards any point of the compass, that the orienting muscles, like the retractor fibres, comprise a cylindrical sheath which, in this instance, consists of short fibres encircling the base of the podium.

VI. LOCOMOTORY STEPPING

During locomotion all the ambulatory feet step in the line of advance of the starfish. The form of the locomotory step is shown in Text-fig. 7A. A retracted podium first orients with its tip thrust forward (A1). The foot then protracts and, when fully extended (A2), swings back through an angle of about 90° with the sucker pressed against, but not firmly attached to, the substratum. This pendulum-like movement has the effect of thrusting the base of the foot forward relative to the sucker, and the sum of the forward thrusts of all the stepping feet determines the movement of the animal as a whole. At the end of the backswing (A3) the sucker detaches and the foot is withdrawn. During the withdrawal movement the foot re-orientates so as again to point in the forward direction (A1).

The step is thus a cyclical activity, comprising a series of linked movements. Ideally it should consist of four movements—protraction, swing back, retraction, and swing forward—alternating with four transitory phases of static

posture (Text-fig. 7, B1–B4). Each posture would be characterized by the static contraction of one member of each of the two opposing pairs of muscles engaged in the execution of the step, namely, the protractor–retractor series and the diametrically opposed protagonists and antagonists of the orienting ring, the four postures representing the four possible combinations of action of the two pairs of opposing muscles. These conditions are set out in Table I below, muscles in contraction being represented in the larger, and relaxed muscles in the smaller, type.
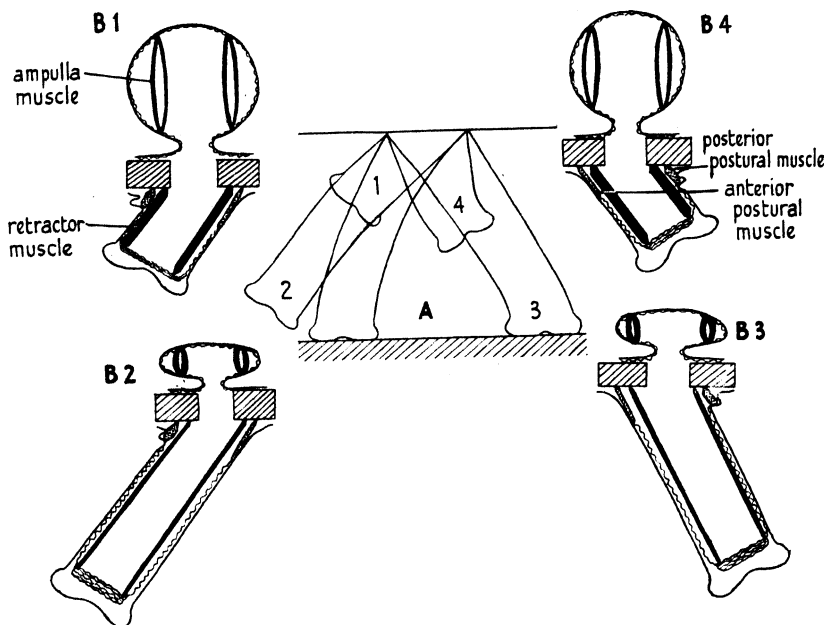
TEXT-FIG. 7. A1–A4. The successive phases of the ambulatory step. B1–B4 show the conditions of contraction and relaxation of the protractor, retractor, and postural muscles of the foot during the successive phases of static posture of the 'ideal' step; the protractor and retractor muscles are shown in black, the orienting (postural) fibres are stippled. The anterior postural fibres orient the foot in the forward direction, the posterior fibres in the backward direction of the step.

As the arrows in Table 1 indicate, each phase of static posture ends, and a new movement begins, with a reversal of the contraction–relaxation relationships of one of the two pairs of opposing muscles, the stepping cycle comprising, in its ideal form, a regular alternation of such changes, first one and then the other pair of muscles being affected.

The actual locomotory step departs from the idealized cycle in certain particulars. As has already been noted, the phase of posteriorly oriented protraction (Text-fig. 7, B4) is omitted: the foot in passing directly from phase 3 to phase 1 combines the movements of withdrawal and of anterior orientation.

TABLE I. *Static phases of the 'ideal' step**

Anteriorly oriented retraction. Phase B1.	Anteriorly oriented protraction. Phase B2.	Posteriorly oriented protraction. Phase B3.	Posteriorly oriented retraction. Phase B4.
ANTERIOR POSTURAL M.	ANTERIOR POSTURAL M.	Anterior postural m.	Anterior postural m.
Posterior postural m.	Posterior postural m.	POSTERIOR POSTURAL M.	POSTERIOR POSTURAL M.
RETRACTOR FOOT.	Retractor foot.	Retractor foot.	RETRACTOR FOOT.
Protractor foot (Ampulla m.).	PROTRACTOR FOOT.	PROTRACTOR FOOT.	Protractor foot.

Phases of movement of the 'ideal' step.

Protraction B1–2	Swing back (Effective step) B2–3	Withdrawal B3–4	Swing forward B4–1

In the actual step.

Tendency for protraction to be delayed.	Marked tendency for swing back to be delayed.	Withdrawal and swing forward occur simultaneously. Phase B4 omitted.	

* The phases of static posture B1–B4 correspond to B1–B4 of Text-fig. 7. Movements are represented by their initial and terminal postures, e.g. B1–B2.

Evidently, at this time, two sets of muscles—the retractor muscles of the foot and the anterior postural muscles—are in simultaneous contraction. Secondly, the smooth rhythm of the step is liable to become dislocated. There is a tendency for the foot to remain poised, often for some seconds, either in the position of anteriorly oriented retraction (B1), or (more frequently) of anteriorly oriented protraction (B2), the podium hesitating either to begin its movement of protraction or to initiate the backswing. These three modifications of the idealized cycle are explicable on the assumption that in each of the two series of opposing muscles one member of each pair is more readily excited to contract than is its partner. Thus, in the protractor–retractor series, the foot muscles are more readily excited than are those of the ampulla while, in the orienting ring, the anterior fibres respond more readily than their posterior antagonists. Some implications of these events will be discussed in a later paper when the nervous mechanisms underlying stepping activity are examined.

SUMMARY

1. An account is given of the muscular anatomy of the foot and ampulla of *Asterias rubens*. An intrinsic musculature of the sucker figured by Cuénot (1891) and Chadwick (1923) is shown not to be present; on the other hand, postural muscles responsible for orientating the podium, levator fibres which 'cup' the sucker, and radial fibres which flatten it are described and figured for the first time.

2. The role of the different muscle systems, the collagen connective tissue, and the fluid of the hydrocoel in protracting and retracting the foot, and in effecting the attachment and detachment of the sucker, is discussed.

3. Evidence is presented to show that postural pointing of the foot is brought about by the contraction of a ring of muscles encircling the base of the podium. The orienting muscles are functionally, but not anatomically, distinct from the longitudinal fibres of the retractor sheath.

4. The ambulatory step is shown to comprise a series of linked phases of static posture and of movement. Each phase is characterized by the contraction of one member of each of the two opposing pairs of muscles engaged in the development of the step. The two pairs of muscles are (1) the anterior and posterior orienting fibres, and (2) the protractors and retractors of the foot. In its ideal form the step comprises four phases of static posture alternating with four movements. Each movement is ushered in by a reversal of the contraction–relaxation relationships of one of the two pairs of opposing muscle systems. Four such changes are possible and they occur in a sequence that ensures the orderly succession of the four movements of protraction, swing

back, retraction, and swing forward, of which movements the idealized stepping cycle is composed.

5. The actual locomotory step departs from the ideal form in two respects: (1) it is liable to become disrupted by a delay in the initiation of the protraction or of the backswing movement, and (2) withdrawal of the podium occurs simultaneously with its re-orientation in the forward direction. It is pointed out that these variations are explicable on the assumption that, in the two series of opposing muscle pairs, the retractor fibres are more readily excited to contract than are their antagonists, and the anterior postural muscles than the posterior postural fibres.

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The Nephridia of *Geonemertes dendyi*

BY

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With five Text-figures

GEONEMERTES DENDYI (Dakin) is a terrestrial nemertine. The genus has a predominantly Australian distribution, but in recent years this species has been recorded from the western parts of England and Wales (Waterston and Quick, 1937; Pantin, 1944). In this genus the nephridial system is highly developed, and its structure in several species has been described by Hett (1924), Coe (1929), and others. Stammer (1934) mentioned the existence of a nephridial system in *G. dendyi* but did not describe it. The present work arose from a study of this species collected in South Devon, in all stages from the newly hatched young to the adult. The nephridial system proves to be at least as highly developed in this as in other species, and there are points of some interest regarding its function.

METHODS

The flame-cells and the proximal part of the tubule system into which they open are easily detected in living worms owing to their ciliary activity, particularly in newly hatched larvae (about 1 mm. long) and immature specimens of about 3-4 mm. in length. The distal parts of the tubule system show no signs of ciliary activity and have not yet been observed in life.

The system is best seen if the worms are immersed in tap-water and slightly compressed with a large coverslip, or held by a small square of wet cellophane covering a shallow hanging drop on the underside of a coverslip above a moist chamber. For prolonged observation under a powerful light, absorption of heat-radiation entering the condenser is essential.

For micro-anatomical studies the best fixatives were found to be Susa, and Zenker in which formic acid is substituted for acetic. Brief preliminary anaesthetization with 5-10 per cent. ethyl alcohol had no apparent ill effects and enabled the specimen to be fixed without distortion. This was done by arranging the animal on a waxed coverslip, covering it with a piece of moist cigarette paper, and dropping it face down on the fixative. Convenient stains were Mallory's Triple Stain, 'Azan', and Heidenhain's Iron-Haematoxylin with or without Masson's Ponceau-Light Green used as counterstain. Paraffin sections 4μ-10μ were cut, commonly after Peterfi's celloidin-paraffin method.

<div style="text-align:center">STRUCTURE</div>

In general the nephridia of *G. dendyi* conform to the plan described by Hett (1924) in *G. hillii*, and by Coe (1929) in *G. agricola*. The flame-cells are exceedingly numerous beneath the dermal musculature. The terminal ducts from groups of them unite in a common canal, the 'end-canal', which passes to a glandular tubule from whence goes a final efferent duct to one of a
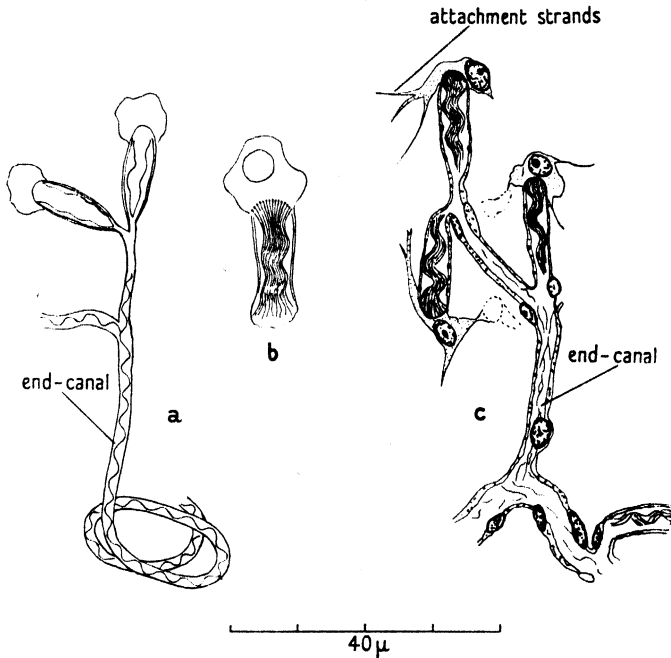
<div style="text-align:center">TEXT-FIG. 1.</div>

A. Living flame-cells and end-canal with helical ciliary wave. B. Isolated active flame-cell from squashed specimen, showing basal granules and partial fraying of end of ciliary flame. C. Flame-cells and end-canal: Formic-Zenker and iron haematoxylin.

very large number of external openings of the system, scattered all over the body.

Text-figs. 1 A, B, and C illustrate living and fixed flame-cells of *G. dendyi*. In fixed material the terminal chamber is $12-17\mu$ long by $3\cdot5\mu$ broad, thus being slightly smaller than those of *G. agricola*, but decidedly larger than those in *G. hillii*. Active living flame-cells are considerably broader than fixed ones, averaging about 17μ by $4\cdot5\mu$. Though it is difficult to measure them alive except during activity, their size seems to diminish somewhat when the ciliary flame stops moving, and their smaller size when fixed may not therefore be entirely an artifact of fixation.

The head of the cell is occupied by a mass of protoplasm which in fixed material appears to be attached to neighbouring structures in the parenchyma by two or three fine strands. As in *G. hillii*, but unlike *G. agricola*, there is only one nucleus in the head of the cell and none in the wall of the terminal chamber. Indications of two or three thickened bands may be seen encircling the terminal chamber, but they do not seem to be as evident as those in *G. agricola*.

In life the terminal chamber is somewhat flattened in a plane parallel to the surface of the worm. Within this tube and lying in the same plane is the powerful tongue-like ciliary flame. Like the flame itself, the sides of the chamber are roughly parallel until near the tip, where they converge to its end-canal.

A clearly defined cushion of protoplasm caps the head of the chamber. Along this cap is a row of basal granules from whence arise the long individual cilia which compose the flame (Text-fig. 1 B). In life the cilia are fused together, though they gradually separate under adverse conditions, a ragged separation first appearing at the tip. The living 'flame' is highly refractile and easily seen.

During activity, sinusoidal waves of contraction pass down the tongue-like flame with a frequency which varies from just above the flicker rate (*c.* 10 per sec.) down to about two a second. At any moment, two to three complete waves are in progress along the flame, their amplitude being about half a wavelength. Under conditions to be described later, the flame may gradually stop. As it does so, the frequency falls off without any change in wave-length or at first in amplitude. On cessation of the beat, the waves may be left for a moment 'standing' and then disappear, leaving the flame extended. As this condition is approached, waves may cease to be propagated along the whole length of the flame.

The active flame almost fills the terminal chamber when viewed in breadth. Viewed sideways, the flame does not fill the chamber when at rest, but during activity the crests of the waves seem to do so, a fact of importance in considering the mechanical efficiency of the flame-cell. At rest, the flame extends straight down the chamber, as it often does after fixation in 70 per cent. ethyl alcohol. Fixation in Susa and Zenker leaves the flame in wave form, often exaggerated.

The staining reactions of the flame in some ways resemble those of the nearby muscle-cells rather than those of the cilia of the ectoderm. It has a strong affinity for acid fuchsin, and the dark colour of iron haematoxylin is retained nearly as long as in the muscle during differentiation.

Text-fig. 2 shows the relation of the flame-cells to the rest of the nephridium. The flame-cells are usually in pairs, the terminal ducts of which join after a short distance (Text-fig. 1). A branched end-canal receives the ducts of several pairs and ultimately passes into a glandular duct. Terminal ducts and end-canals have moderately thin walls with scattered nuclei but without apparent cell-boundaries. In *G. agricola* the end-canals open directly into

the glandular part of the nephridial duct, which is convoluted (Coe, 1929). In *G. hillii* they are in addition frequently coiled (Hett, 1924). In *G. dendyi* the end-canals appear to be more highly organized. Branches from the terminal ducts of the flame-cells unite to form a canal which commonly runs
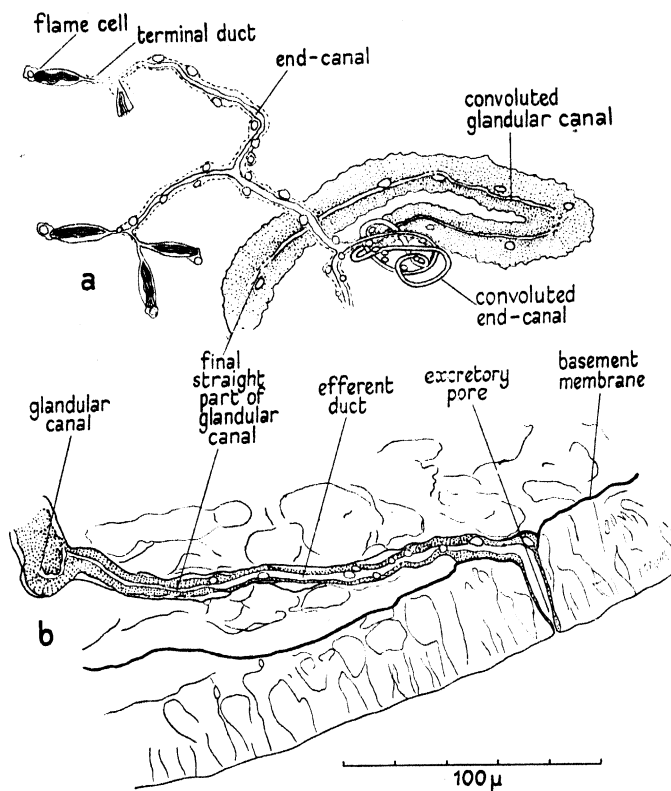
TEXT-FIG. 2. Camera lucida outlines.

A. Flame-cells, end-canal with convolution, glandular canal.

B. Glandular canal, efferent duct, excretory pore.

fairly straight for a considerable distance. As it does so the canal widens from about 2 μ to 4 μ in diameter, and the walls thicken slightly. The canal then undergoes two or three convolutions, after which it passes into a thick-walled glandular part of larger cross-section but of rather smaller lumen. This glandular part generally continues the convolution (Text-fig. 2 A). The convoluted portions of both end-canal and glandular duct are contained in well-defined spaces in the parenchyma. During life the end-canal is easily seen owing to the ciliary movement. This is absent in the terminal ducts. The movement begins in the branches of the end-canal and becomes stronger as

the convolution is approached. Ciliary movement in the convolution presents a striking appearance. It seems to finish abruptly at about the point where sections show the glandular region to begin. Fixed material shows that the lumen of the end-canal carries long fine helical fibrils, apparently cilia (Text-fig. 3 A).

The action of the cilia gives the appearance of helical waves running rapidly down the end-canal and round its convoluted portion. That the motion is
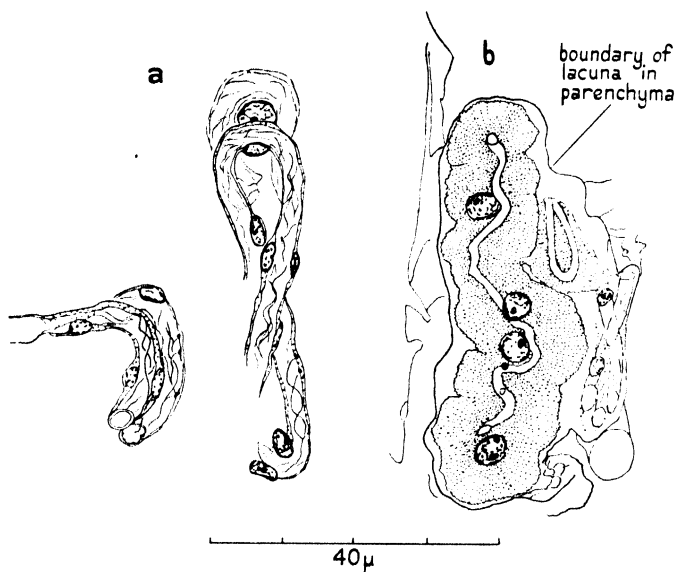
TEXT-FIG. 3. Camera lucida outlines.

A. Convoluted end-canal with cilia.   B. Part of glandular canal in lacuna of parenchyma.

helical, and not a plane wave as in an undulating membrane, is apparent from the fact that it can be seen from whatever angle the canal is viewed. The appearance (Text-fig. 1 A) resembles that seen in the nephridial ducts of Lumbricus.

No cilia can be seen beyond the convoluted end-canal in life, and none can be detected in sections of the glandular canal or efferent duct to the exterior. The walls of the glandular canal are thick and granular and have the well-marked radial structure recorded by authors in other species (Text-fig. 3 B). In poorly fixed material the radial fibres actually separate, giving the tubule the appearance of a contorted bottle-brush. As in other species, there are scattered nuclei, but no cell boundaries are evident. Evidence will be presented later that the end-canal with its convolution and the glandular canal are physiologically distinct.

The glandular canal finally leaves the lacuna of the parenchyma occupied by the convolution, and may run some distance in a fairly straight path

(Text-fig. 2 A and B). The histology of the duct then changes again to that of a thin-walled tube. This, after running just beneath the basement membrane, abruptly enters one of the numerous excretory pores. These consist of a hollow flask-shaped cell resting on the basement membrane and opening to the exterior by a minute pore (Text-fig. 2 B). How numerous these pores are is shown in Text-fig. 4, taken from a tangential section through the dorsal
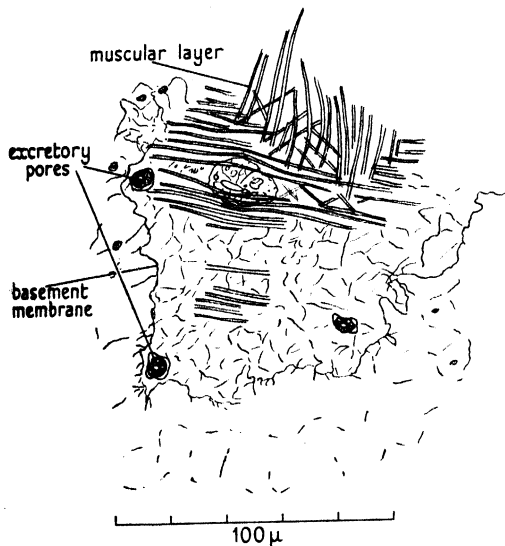
TEXT-FIG. 4. Tangential section through dorsal surface.

surface and basement membrane. Over much of the surface they are only 50–100 μ apart.

ANATOMICAL RELATIONS

The body of *G. dendyi* differs from that of most marine metanemertines in the thinness of the muscular body-wall and the voluminous parenchyma. The latter consists largely of connective tissue divided into well-defined lacunae and channels containing structures such as the convoluted tubules of the nephridia and the blood-vessels. It is this tissue which varies most clearly in volume according to the water content of the animal.

The thin muscular layer lies immediately below the basement membrane, and among and immediately below the innermost muscle-fibres, the longitudinal ones, lie the flame-cells. In *G. dendyi* they are confined to this plane and do not seem to occur in other situations, such as below the surface of the rhynchocoel. They are most numerous over the head and along the sides of the body. They are fewer along the mid-ventral line, and fewest along the mid-dorsal line, above the rhynchocoel. Text-fig. 5 A shows the distribution

of flame-cells on the upper surface of a newly hatched (1 mm.) larva. As the animals grow, the relative number of flame-cells increases even more rapidly, so that over the head and sides no point on the surface of the animal is more than 10–20 μ from one of these structures. Water entering or leaving the body must pass near one or more of them.
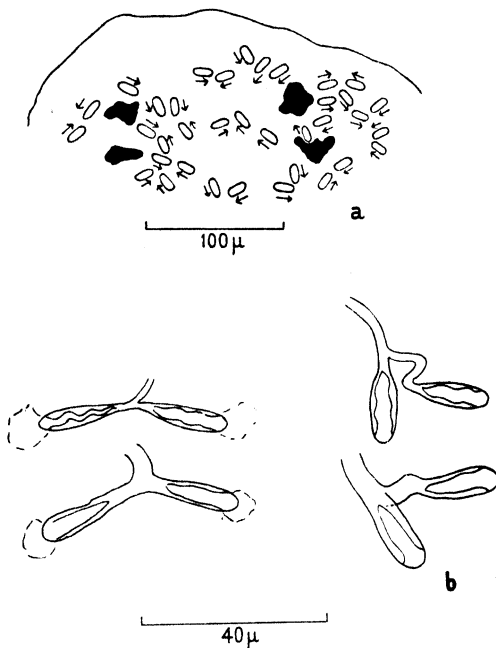
Text-fig. 5.

A. Head of immature larva showing distribution of active flame-cells: arrows show direction of flame. B. Sketches of flame-cells showing swelling of terminal ducts after compression.

Below the muscular layer and among the nephridia there ramifies a network of fine contractile blood-vessels, derived from the lateral vessels. As Coe (1929) points out in other species of Geonemertes, so also in *G. dendyi* the flame-cells are not in intimate contact with the vessels, as they are in some marine nemertines; they are, however, always near them and occupy the same stratum below the muscle layer.

The convoluted tubules in their lacunae of the parenchyma lie distinctly deeper than the flame-cell-vascular layer. The efferent ducts running out from these ultimately make their way again to the surface.

FUNCTION OF THE PROTONEPHRIDIA

Since the time when Goodrich (1899) made the suggestion that the solenocytes of *Glycera* are functionally analogous to the Malpighian corpuscles of

the vertebrate kidney and excrete fluid, the analogy has been extended to other protonephridia, particularly in Platyhelminthes (Reisinger, 1922; Westblad, 1922). Apart from the fact that the flame-cell system of freshwater Turbellaria is much better developed than that of their marine relatives, there is direct evidence in various acoelomates that water is excreted by such systems. Water can be seen to accumulate in the contractile bladders of some Rhabdocoela, Trematoda, and Rotifera, and to be periodically emptied. Herfs (1922) showed that in certain cercariae the rate of water secretion decreased as the osmotic pressure of the external medium was increased.

In *G. dendyi* there is no doubt that the upper part of the protonephridial system secretes fluid. If a relatively transparent individual is left for a time in fresh water and then compressed under a coverslip, the flame-cells and the ducts leading immediately from them can sometimes be seen to swell during the course of 10–15 minutes (Text-fig. 5 B). This is most often to be seen in the flame-cells that are farthest from the edge of the animal, and are therefore the most subject to compression.

Observation shows that there is a relation between excretion of water and the activity of flame-cells. During life this is often intermittent, or the cells may even be inactive for long periods. No swelling of the tubule system has so far been seen except when the ciliary flame is active. Moreover, the activity seems to be related to the water-content of the internal medium. It is absent in animals that have been allowed partly to dry, but is resumed if the animal is immersed for a time in tap-water. If an animal showing intermittent activity of the flame-cells is examined under a coverslip, it is seen that local groups of the cells begin to be active more or less simultaneously. Later, all gradually stop, though some continue to beat many minutes longer than others. Renewed activity often promptly follows local movements in the worm, either contraction of the body or the peristalsis of a blood-vessel in the neighbourhood of the flame-cell. Immobility of the worm is always followed sooner or later by inactivity of the ciliary flames, except those on the edge where the body is in contact with water under the coverslip. Activity of the flames can often be engendered by slightly raising and lowering the coverslip—as when focusing an oil-immersion lens when the coverslip is unsupported. Thus anything which tends to stir the internal medium round the flame-cells leads to the activity of the flame, while stagnation of the medium is correlated with inactivity.

If an animal with active flames is carefully squashed under a coverslip, it is possible to reach almost complete disintegration of the tissues while the individual flame-cells still beat for a time. If when they cease to be active, tap-water is allowed to run under the coverslip, the flames restart—even though they sometimes appear to be more or less isolated from neighbouring cells and detritus. It is possible to revive the activity of the flame-cells in the squashed mass by irrigation several times in succession. It is even possible sometimes to revive them by lifting the coverslip with some adhering cell debris and flame-cells and remounting it in a fresh drop of tap-water. It is

remarkable that the flame-cells should be able to resume or to continue their activity after breakdown and removal of much of the surrounding tissue.

DISCUSSION

It is difficult to avoid the conclusion that dilution of the internal medium of the parenchyma is a stimulus to the activity of the flame-cells. And since under conditions of activity water appears to pass into the nephridial system, it is natural to suggest, in conformity with common opinion, that the ciliary flame actually drives water into the system. But Carter (1940) has pointed out a difficulty in assuming the mechanism is powerful enough for this purpose. Ciliary and flagellar mechanisms elsewhere in the animal kingdom have not been shown to be able to produce large differences of pressure. Parker (1919) found the oscular pressure of the sponge *Stylotella* to be 3·5–4 mm. of water. Such pressures would be wholly inadequate for the separation of water from the salts in the body fluid. But they might suffice to overcome a very low colloidal osmotic pressure (cf. Picken, 1937), and Reisinger (1922) suggested that in the excretion of water by flame-cells the colloids of the body fluid might be kept back. The mechanical plan of a flame-cell seems to be far more efficient than that of the flagellate chamber of a sponge for the creation of a pressure-difference.

It has been suggested (Carter, 1940) that the cilia of flame-cells may not function as a pump, but rather as a stirring mechanism to prevent accumulation of substances which would hinder a process of active secretion. But since fluid is passing into the tubule system it seems unlikely that there could be such an accumulation on the inner boundary of the terminal chamber.

While it has not been possible to obtain a direct demonstration that the ciliary flame in *G. dendyi* is responsible for the flow of fluid into the tubule system, the observations recorded here certainly suggest this. Due weight must be given to the fact that if a ciliary mechanism can exert the necessary pressure-difference at all, the mechanical design of a flame-cell, as seen in operation, seems admirably adapted for such a purpose. Further, there is evidence that some pressure at least is set up by the action of the flame. If the tissues of an animal are gently squashed under a coverslip so as partly to separate the flame-cells from the surrounding tissues, the action of the flame sometimes causes visible oscillations of the base of the cell. Since the flame is apparently free inside the terminal chamber, the oscillation is presumably due to pressure-differences set up within the chamber.

Whatever the part played by the ciliary flame in the process, the evidence suggests that the nephridia of *G. dendyi* excrete water. But though it is easy to relate the development of the nephridia of freshwater acoelomates to an inevitable osmotic intake of water from the medium, the existence of a mechanism for excreting water in a terrestrial animal is remarkable. Such an animal might be expected to provide against loss of water rather than for its excretion. Loss of fluid in *G. dendyi* may certainly be great. The worms normally range from 1 to 20 mm. in length by 0·5 to 3 mm. in breadth. They

continually secrete a tube of mucus in which the animal moves forward during locomotion, through the action of cilia which completely cover the body. Loss of mucus and evaporation cause the body visibly to diminish in size when exposed to an unsaturated atmosphere. When unable to escape to a moister place the animal ceases to move and the outer mucus hardens to a transparent cocoon in which water loss appears to diminish. Such cocoons, however, are sometimes formed to provide an aqueous medium for the eggs which are shed into them, together with a quantity of fluid. This is a further tax on water reserves, though the eggs are normally laid in very damp situations, where there may even be a film of running water. But the most rapid loss of water takes place when the proboscis of the worm is everted. This is primarily a flight response in this species, and when it occurs so much mucus is lost that after three or four eversions the animal becomes visibly smaller.

But while water loss may sometimes be considerable, it is incorrect to suppose that desiccation operates continuously. The worms live under damp stones and logs, particularly in woods where low vegetation impedes the drying action of the wind. They possess strongly developed responses, particularly positive geotaxis and negative phototaxis, which remove them from exposed situations. In a gradually drying vivarium they can be seen to bury themselves. Though sometimes to be found far from fresh water, they may be taken from under a log, of which the other end lies in a film of water sufficient to support *Planaria vitta*. They are often found in contact with dew or rain-water, and survive immersion in fresh water for some days, though not indefinitely. The worms are, in fact, frequently exposed to pure water: a situation more exacting than that to be met by many freshwater organisms. Their natural conditions of existence are thus not those of continuous desiccation, but may vary between this and excessive hydration. Hence any regulating mechanism must be able to deal with extremes of water conditions. The evidence presented here shows that the nephridial system can eliminate excessive water, and that under conditions of water loss the ciliary flames become inactive. What part is played by the highly differentiated portions of the nephridial canal remains to be seen.

I wish to express my gratitude to Mr. O. D. Hunt and Mr. B. W. Sparrow for much help in the collection of specimens.

SUMMARY

1. The nephridia of the terrestrial nemertine *Geonemertes dendyi* are described. The flame-cells are in groups, each of which communicates with the exterior by its own duct. The flame-cells open into the branches of a ciliated end-canal. Distally this end-canal executes a number of convolutions and then passes into a non-ciliated glandular canal. The first part of the glandular canal is also convoluted, and the distal part of it passes to the efferent duct.

2. The nephridia are distributed over most of the surface of the animal immediately below the muscular layer. The convolutions of the end-canals

and glandular canals lie somewhat deeper, in well-defined lacunae in the parenchyma.

3. Evidence is presented that fluid can be excreted into the nephridia. The activity of the flame-cells varies with the degree of hydration of the animal and may cease on desiccation. The flame-cells can be stimulated to activity by dilution of the body fluid of crushed animals with tap-water. The relation of water excretion to the mechanical activity of the flame-cells is discussed.

4. Some features of the natural habitat and the water relations of the animal under natural conditions are described. It is pointed out that the animal may have to contend not only with desiccation but with excessive hydration. The physiological consequences of hydration appear to be met by the flame-cell mechanism.

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The Behaviour of Mammalian Skin Epithelium under Strictly Anaerobic Conditions

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With eight Text-figures

IN the course of some experiments on the nature of skin transplantation immunity, it became necessary to find out for how long (if at all) mammalian skin epithelium can survive in the total absence of atmospheric oxygen. The results of the investigation are summarized here in the hope that they may be useful to other students of tissue behaviour.

METHODS

Principle. Small squares of thinly sliced skin are removed from the body, placed in an adequate quantity of a 'physiological' fluid, incubated in an atmosphere of hydrogen, and then, after varying periods, returned by a grafting operation to the animal from which they originally came. The outgrowth of epithelium from such grafts constitutes a proof of their survival that is hardly subject to error (Medawar, 1944).

Preparation of the skin slices. Rabbits' skin has been used throughout. The skin of the chest or belly is shaved 4 days before the experiment, in order to toughen the otherwise very fragile cuticular layer and to thicken the epidermis. It is undesirable to use so-called 'pigmented' skin or skin of the spongy hyperaemic type that grows hairs very quickly. Skin of this active type may have an abnormally high metabolic requirement (cf. Peyton Rous, 1946).

On the day of operation, the rabbit is anaesthetized with ether and the shaved skin area washed for one minute with a cotton-wool swab soaked in a $1\frac{1}{2}$ per cent. solution of cetyltrimethyl-ammoniumbromide ('CTAB', 'Cetavlon', a cationic soap: Barnes, 1942). The soap is removed with a fine jet of surgical spirit, and the skin surface at once dried with sterile swabs and very thinly coated with sterile vaseline applied with a glass rod. A central part of the area is divided into a grid of squares of side 3 mm. by very light scalpel incisions; the squares are then sliced off by using a 'Durham Duplex' razor blade firmly clamped in stout Spencer-Wells forceps, or a newly stropped straight-edge scalpel blade (e.g. Swann-Morton No. 11). After cutting, the grafts are placed raw side down in a Petri dish containing a circle of filter paper that has been damped with Ringer's solution.

Preparation of the incubation media. Serum is prepared from the skin donor by withdrawing 10–30 ml. blood from the median artery of the ear,

previously washed, shaved, and rendered hyperaemic by rubbing with a little xylene. The blood is allowed to clot spontaneously and then briskly spun. The saline solutions that replace serum in some of the experiments described below are Krebs-Ringer-bicarbonate or Krebs-Ringer-phosphate, in each case containing a final concentration of 1 : 100,000 phenol red and 20 u./ml. streptomycin (see below). The 'iodoacetate' referred to below is prepared by dilution of an M/100 solution made up as follows: 0·465 g. monoiodoacetic acid is dissolved in about 200 ml. water containing 2·5 ml. 1 : 1000 phenol red. 2·50 ml. N/1 NaOH solution is run in slowly, and the volume made up to 250 ml. by the addition of further water.
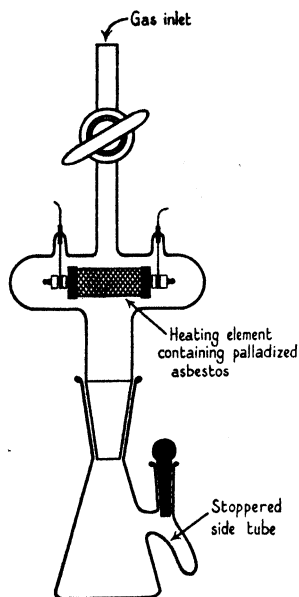
TEXT-FIG. 1. Anaerobic incubation apparatus.

Technique of incubation. The 'cultivation' of the skin squares is simply a matter of floating them raw side down, in pairs, on 5 ml. culture fluid in standard 50-ml. conical flasks with B-19 size ground-glass stoppers. In 'aerobic' control experiments the gas medium is simply air. It is desirable, but not absolutely necessary, that the flasks should be shaken from side to side during the run of the experiment by a slow tilting or rocking motion; and also desirable, but again not necessary, that the flasks should be incubated in a 38° C. water bath rather than in a bacteriological dry air incubator.

The technique for anaerobic incubation requires the fitting of a similar conical flask with a stoppered side tube (as in manometric apparatus) to the 'unit' illustrated by Text-fig. 1. The principle of anaerobic culture is to grow the tissues in an atmosphere of cylinder hydrogen from which the last traces of oxygen have been catalytically removed by a heating element wrapped around asbestos impregnated with metallic palladium. This technique was introduced in bacteriology by Laidlaw (1915), and the 'element' illustrated in Text-fig. 1 is the standard fitting supplied with McIntosh-Fildes's anaerobic culture jars. The side tube of the culture flask contains an oxygen indicator.

The following is a step-by-step account of a method that has given satisfactory results. Five ml. culture fluid are put into the culture flask, followed by the two skin squares. The side tube is then filled with about 1 ml. indicator solution freshly mixed from the following stock ingredients: 1 : 1000 methylene blue, 2 ml.; 1 per cent. glucose in water, 8 ml.; N/1 NaOH, 5–7 drops. (It might seem preferable to use an indicator with a more negative oxidation-reduction potential, such as neutral red reduced with sodium hyposulphite ('hydrosulphite', $Na_2S_2O_4$); but this system is difficult to work

with, and since the indicator is meant to reveal leaks and not to measure O_2 tension, the methylene blue is perfectly satisfactory.)

The loaded culture flask is attached to the unit, and H_2 gas from a cylinder with a reduction valve and water trap is passed briskly through the apparatus for 2 minutes. The gas escapes through the side-tube stopper, left slightly open. The flow is then reduced to a trickle, the side-tube closed, and current passed through the terminals of the heating element, using 60-W. and 40-W. gas-filled light bulbs in parallel as resistances. Water is soon seen to condense on the glass housing the element. Half an hour's passage of current is more than sufficient; the top tap is closed, the current switched off, and the whole apparatus incubated at 38° C. The indicator becomes white after an hour or so of incubation. It will do so earlier if the NaOH concentration is raised, but this is unnecessary.

At the end of the experiment the skin slices are rinsed in Ringer's solution; one is grafted back to its donor, and the other kept for histological examination. When air is admitted, the heating element will be seen to 'steam' and the indicator to turn blue.

Note on asepsis. All the operations described above were done aseptically; but skin cannot be wholly freed from its 'resident flora', no matter how much care is taken, so there is always a danger that the experiment may be ruined by infection. Streptomycin at a final concentration of 20 u./ml. in the culture medium has been found to abolish this danger: it is not visibly harmful to skin epithelium at ten times this concentration, and has now been used for two years as a routine measure in all the writer's tissue-culture experiments with skin. (The sample used (1 mg. = 250 u.) was kindly supplied by Dr. E. S. Duthie, who suggested its use for tissue-culture purposes (cf. also Heilman, 1945). Streptomycin, discovered by S. A. Waksman (cf. Waksman, 1945) is an antibiotic base recovered from the soil fungus *Actinomyces* (*Streptomyces*) *griseus*: unlike the acidic penicillin, it is active against Gram-negative organisms.) It has proved convenient to prepare a solution containing 200 u./ml., sterilized by passage through a bacteria-retaining sintered glass filter, and to dilute it before use with nine times its volume of the culture medium.

The test of survival. Tissue-culture itself offers one obvious method by means of which the viability of cells presumed alive or dead may be put to the test. For testing the viability of skin, however, transplantation technique is in every respect (and above all, in sensitivity) much superior. The skin squares are rinsed in Ringer's solution and, after aerobic cultivation only, their dermal surfaces are gently scraped to remove the epithelium that will have grown over and encysted them. The rabbit that provided the grafts is then shaved over the right side of the chest, and the skin so exposed rubbed with 5 per cent. aqueous 'Dettol' solution, which is allowed to dry on. (It is possible but not very desirable to use a rabbit other than that which provided the skin squares. As the grafts are small, and represent a fairly low 'dosage' of foreign homologous skin, they can be expected in the majority of cases to survive and proliferate during the 10-day test period without serious interference from the

homograft immunity reaction (cf. Medawar, 1944, 1945).) A square of skin of 5 cm. side is then stripped off the sterilized area in the natural 'splitting' plane that lies between the panniculus carnosus beneath and the compact collagenous tissue of the dermis above. Every tag of skin that might house the base of a hair follicle must be removed—a very easy operation, unless the skin is unusually thick or the incision insufficiently radical. The skin grafts are blotted dry on sterile gauze and laid raw side down on the centre of the raw area, which is then thickly dusted with sterile sulphadiazine powder,


TEXT-FIG. 2. Part of a 10-day test graft showing full survival: note the outgrowth over the raw area of thick hyperplastic epithelium.

covered with a widely overlapping square of vaseline-impregnated gauze ('tulle gras'), and a square of several thicknesses of dry gauze. The thorax is thereupon firmly wound, first with 1 yard of 3-in. bandage, and then with 18 in. of plaster-impregnated 4 in. bandage (e.g. 'Gypsona'). After the plaster has dried, the animal requires no further special attention until the inspection 10 days later.

If the graft proves at this inspection to be surrounded by an annulus, 2–5 mm. wide, of thick and glistening white epidermal epithelium that has spread from it over the surrounding raw area, the result of the experiment may be recorded as 'survival' without further ado. In any other event the graft should be removed and examined by sections cut at four or five levels and stained with Ehrlich's haematoxylin and eosin. If only a trace of follicular epithelium remained alive in the original skin square, it may not have had time during the 10-day test period to do more than proliferate locally and force its way to that part of the graft surface that immediately overlies it. Naked-eye inspection alone hardly makes it possible to distinguish between this state of affairs and that in which the graft fails altogether to survive.

Text-figs. 2, 3, and 4 illustrate the histological appearances of test grafts showing full survival, partial survival, and no survival respectively. Each was

removed 10 days after transplantation. The first two yield a positive result—survival—and this can hardly be subject to error. The third result—non-
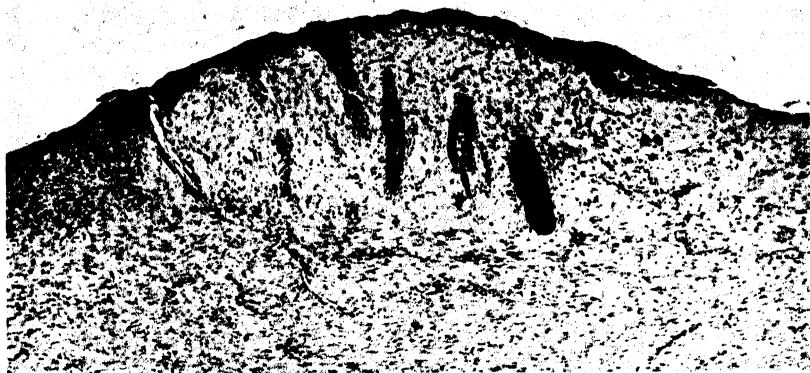
TEXT-FIG. 3. Ten-day test graft showing partial survival. Follicle epithelium has forced its way to the graft surface, but there is no outgrowth over the raw area.


TEXT-FIG. 4. Ten-day test graft showing no survival. The dermis of the graft is deeply embedded in the granulation tissue into which the surrounding raw area has become transformed. There is no surviving epithelium.

survival—must be subject to a small and consistent error; for if only a dozen or so cells remain alive in the incubated skin square, they may not be able to survive the further short period of ischaemia entailed by the grafting operation.

RESULT

(A) *Behaviour of skin incubated aerobically in serum.* Skin squares incubated for 4 days in 4·5 ml. autologous serum + 0·5 ml. streptomycin 200 u./ml. show survival and proliferation of all epidermal elements and a strong migratory overgrowth (epiboly) round the dermal pad that bounds the skin square below. In consequence, the skin square encysts itself in epithelium (Text-fig. 5). Growth is better in a stirred medium, and better still if blood corpuscles are added in physiological concentration to the serum to give an artificial non-clotting 'whole blood'. A full description of such skin cultures
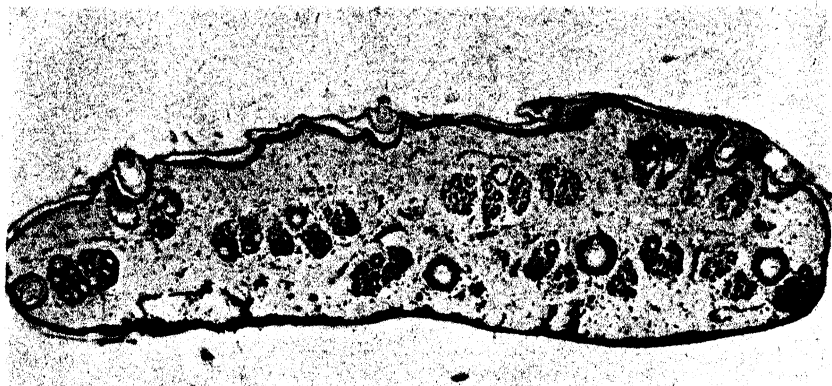
TEXT-FIG. 5. Skin square incubated aerobically in serum for 4 days. Migratory overgrowth of the epithelium round the dermal surface has brought about complete encystment.

will be published elsewhere; the only conclusions relevant here are that cultures incubated aerobically in serum show full survival, migratory overgrowth, and cell division. (Migratory overgrowth is the equivalent of 'outgrowth' in conventional tissue-cultures.)

(B) *Behaviour anaerobically.* Skin incubated anaerobically for 4 days in serum with streptomycin shows either full survival, as indicated by the grafting test, or something very little short of it. As incubation proceeds beyond the fourth day, the quantity of outgrowth from the test graft progressively falls off; until, by the eighth day, it is reduced to scraps of hyperplastic follicle epithelium, which may have migrated upwards to form a secondary coating of surface epithelium immediately above them (Text-fig. 3). Beyond 8 days of incubation, the test graft shows no survival (Text-fig. 4). However, the addition of extra glucose to the serum (0·5 per cent. above the original concentration) proved in each of two independent trials to prolong survival to the ninth day at least.

Histological analysis of the skin squares, after incubation but before test grafting, was made by fixing one of the two pieces normally housed in each vessel. Skin cultivated anaerobically shows neither cellular movement nor

cell division: the edges of the skin square, instead of being rounded off by epithelial overgrowth, remain as sharp as they were when originally cut with a scalpel blade (Text-fig. 6). A most determined search for mitotic figures in sections stained with Heidenhain's haematoxylin revealed none. There is not even any migration of follicle epithelium towards the skin surface: the hair bases remain firmly gripped in their epithelial jackets

TEXT-FIG. 6. Contrast with Text-fig. 5: the edge of a skin slice incubated anaerobically in serum for 4 days. The slice ends off abruptly; there has been no migratory overgrowth, nor any upward migration of follicle epithelium. The marginal cells of the surface epithelium have undergone maceration, but the follicle epithelium is in a surviving state.

(Text-fig. 6) and the follicles show none of that flare-like enlargement that is indicative of upward migration.

The majority of the incubated skin squares were fixed in $HgCl_2$-formaldehyde; dehydrated and cleared in an ethyl alcohol series, cedarwood oil, and ligroin; embedded in paraffin wax; cut at 8μ thickness; and stained with Ehrlich's haematoxylin and eosin. After this treatment the cells of the deep follicle epithelium present a characteristic and uniform appearance (Text-figs. 7 and 8). The cytoplasm is weakly basiphilic and diffuse or cloudy in general staining reaction. The nuclei, instead of being large and juicy in appearance, give the impression of being withdrawn from the cytoplasm, are irregular if not frankly crenated in outline, and stain rather densely—though not in the fashion described by pathologists as 'pyknotic'. The surface epithelium is much more variable. After short periods of incubation the cells

are typically in the condition just described; but after longer periods they may give the characteristic appearance of aseptic necrosis. The cells may have separated from each other, or the reaction of the cytoplasm may be acidophilic and the nuclei represented by ghost outlines alone. Cells of this appearance are presumably dead; but those described beforehand, though an histologist

TEXT-FIG. 7.   TEXT-FIG. 8.

TEXT-FIG. 7. The follicle epithelium illustrated by Text-fig. 6: higher power. For description, see text. (The sections illustrated in Text-figs. 7 and 8 have been photographed in such a way as to bring the nuclei into the highest possible contrast; their frankly pyknotic appearance is thus largely a photographic artifact.)

TEXT-FIG. 8. Cf. Text-fig. 7: another island of deep follicle epithelium; from a graft incubated anaerobically for 6 days in serum.

might well call them highly abnormal and degenerate, are in fact 'alive' in the sense defined by the grafting test. A cytochemical study of the cells, outside the scope of the present inquiry, might be very revealing.

It appears, then, that cellular survival is sharply dissociable from cell division and cell movement; and that the presence of molecular oxygen is a necessary condition for division and movement, but not for mere survival.

So far as can be judged by histological examination alone, dermal mesen-

chyme cells (fibroblasts, histiocytes, and vascular endothelium) may survive anaerobically for 4 or 5 days. They are not as resistant to anaerobiosis as is skin epithelium, and after 6 or 7 days are reduced to pyknotic or fragmented scraps of nuclear material uninvested by cytoplasm.

(C) *Behaviour in physiological salt solutions.* All the experiments to be described under headings (C) and (D) make use of a 4-day incubation period; a period chosen because after 4 days of anaerobic incubation in serum survival is still complete or falls little short of it.

Skin slices will survive aerobic incubation for 4 days in Krebs-Ringer-bicarbonate or Krebs-Ringer-phosphate, in either case with *or without* the addition of glucose (0·5 per cent. final concentration): they will *not* survive 4 days' anaerobic incubation, without *or with* glucose in the same concentration. (The phosphate-buffered Ringer is the medium of choice for anaerobic experiments of the type described in this paper. The reaction of a bicarbonate-buffered medium becomes progressively more alkaline during the course of the experiment, presumably because of the escape of CO_2 into the hydrogen gas phase.)

(D) *The iodoacetate-sensitivity of skin slices.* Skin incubated aerobically *in vitro* has proved to be very sensitive to iodoacetate. An attempt was first made to determine the threshold concentration of iodoacetate that *just* permits the survival of skin after 4 days' aerobic incubation in a medium containing autologous serum 4·0 ml., streptomycin (200 u./ml.) 0·5 ml., and iodoacetate solution 0·5 ml. The accompanying table sets out the results of 7 independent experiments: '1' stands for 'survival' and '0' for non-survival in the test grafts.

	1	*2*	*3*	*4*	*5*	*6*	*7*
M : 7,500 . . .				0	1	0	0
M : 10,000 . . .	1	1	0	0	1	0	0
M : 12,500 . . .				0	1	0.	1

Five independent preliminary trials at a concentration of M : 4,000 had given uniformly negative results. The results are evidently somewhat variable, though they put the order of magnitude of the minimal lethal concentration at M : 10,000. Part of the variation may be attributable to the presence in serum of a variable quantity of a glycolysis-inhibiting factor (cf. Elliot and Henry, 1946).

More consistent results were obtained from four additional independent tests in which serum was replaced by Krebs-Ringer-phosphate containing 0·5 per cent. glucose, and a slightly wider spacing of iodoacetate dilutions was used:

	8	*9*	*10*	*11*
M : 5,000 . . .	0	0	0	0
M : 10,000 . . .	0	0	0	0
M : 15,000 . . .	1	0	1	1

The three 'positives' recorded under M : 15,000 were not, however, of equal value: in one of the three positive cases survival was 'partial' only; in the other two, considerable but probably not complete. Since rabbit skin varies a good deal in 'activity', i.e. in its intrinsic rate of cell division on removal, it is doubtful if these results justify a more definite conclusion than that the order of magnitude of the minimal lethal concentration of iodoacetate, after 4 days' exposure, is of the order of 10^{-4} molar.

<div align="center">DISCUSSION</div>

The fact that epidermal movement and cell division is brought to a standstill under anaerobic conditions is not unexpected (cf. Needham, 1942; Kitching and Moser, 1940; Ormsbee and Fisher, 1944). It is surprising that the power merely to survive 'vegetatively' at body temperature is so sharply dissociable from migratory and karyokinetic activity; but the experimental results show quite clearly that skin epithelium may survive anaerobically in serum for periods upwards of a week at body temperature. *Paramecium*, so Kitching (1939) found, can live anaerobically only for a matter of hours—though he quotes earlier estimates ranging from a few seconds to a few months!

Skin epithelium is a tissue that depends for its blood-supply upon the capillary circulation in the upper reaches of the dermis. Presumably, therefore, it has to submit to low oxygen tensions as a matter of course, and relies to a large extent upon a glycolytic mechanism. Berenblum, Chain, and Heatley (1940) and Dickens and Weil-Malherbe (1943) find that skin epithelium displays aerobic as well as anaerobic glycolysis; Dixon and Needham (1946), by contrast, find that the abdominal skin of 4-day old rats shows no aerobic glycolysis but metabolizes after the fashion of skeletal muscle.

If it is correct to assume that iodoacetate in a concentration of the order of 10^{-4} molar is a specific inhibitor of glycolysis, then these two definite conclusions may be drawn from the experiments described in the text: the survival of skin epithelium depends upon the integrity of its glycolytic mechanism; but cellular movement and division depend upon respiratory activity in the narrow sense. It may be added, though at present only on the basis of rough observations, that the rate of cell division in skin epithelium varies directly with O_2 tension over a fairly wide range. The behaviour of skin cultivated *in vitro* (to be described elsewhere), and of the cornea *in vivo*, shows that epidermal epithelium does not need much oxygen in order to grow and move. It does, however, need some. It is possible that cartilage cells behave in like manner (Bywaters, 1937).

<div align="center">SUMMARY</div>

1. The viability of skin epithelium subjected to various experimental treatments may be tested by transplantation technique.

2. Skin can survive for upwards of a week in serum at body temperature in the total absence of atmospheric or dissolved oxygen.

3. Neither cellular movement nor cell division can take place in skin epithelium under anaerobic conditions.

4. The survival of skin epithelium depends upon the integrity of a mechanism, presumably glycolysis, that is put out of action by iodoacetate at a concentration of the order of 10^{-4} molar.

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Further Observations on the Glomerular Root of the Vertebrate Kidney

BY

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With five Text-figures

INTRODUCTION

IN the kidneys of a wide variety of animals the second convoluted tubule is applied to the root of the glomerulus of the same nephron (Text-fig. 1). Here the tubular cells are aggregated to form the *macula densa*. In these cells the Golgi element lies on the side of the nucleus opposite to that on which it lies in the rest of the tubule (McManus, 1943, 1944). The tubule is set into the angle between the afferent and efferent arterioles of the glomerulus (Text-fig. 2A). Rotation of the plane of section through 90° produces the appearance shown in Text-fig. 2B. The arteriolar cells in this region are known in a variety of species to possess granules in their cytoplasm.

These relationships become established at a very early stage in embryonic development. In the human foetus, and in every other embryo examined from fishes upwards, a segment of tubule applied to the glomerular root can be recognized as soon

TEXT-FIG. 1. Glomerular complex. Diagram showing relationships at root of glomerulus. From *Lancet*, 1942.

as there is a glomerulus supplied by two vessels destined to become the arterioles.

Since the last review of the structure of the glomerular root was written (McManus, 1942), not a great deal has been published about the problem of the role of the components of the glomerular or juxta-glomerular complex. Goormaghtigh (1944) has continued to present evidence suggesting an endocrine activity for the granular cells, and has recently (1945) described the hyperplasia of these cells in the so-called 'crush' kidney, that is, the human kidney after severe wounds in other parts of the body, especially of the limbs. The present communication describes further findings concerning the cytology and histology of the glomerular root.

RETICULIN PATTERNS AT THE GLOMERULAR ROOT

The distribution of reticulin has been studied by various modifications of Robb-Smith's (1937) method of floating paraffin sections on the solutions used. Foot's technique (1924) was used, as Robb-Smith recommends, as well as Divry's (1932) modification of Hortega's silver carbonate. The use of thinner sections made possible by this 'floating' routine has permitted the recognition of features that are obscured in thicker sections, and especially in thick frozen sections.
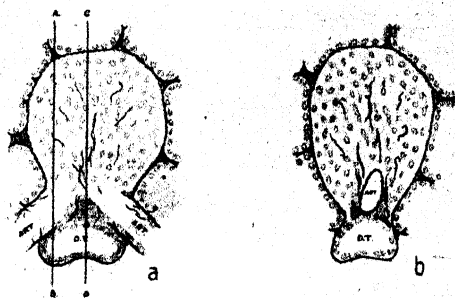
TEXT-FIG. 2. Diagrams of reticulin about glomerulus.
Text-fig. 2B is at plane AB of Text-fig. 2A.

A complete basement membrane, on which the epithelial cells rest, surrounds each tubule and inter-tubular fibrils connect these membrane units with the vascular tree of the kidney. Further subdivisions of the reticulin fibrils have been described by Tello (quoted by Ramon-y-Cajal, 1933), and the development and age-changes have been discussed by Schwab (1939). These authors do not describe the arrangement of the reticulin at the glomerular root.

I find that there is always a gap in the reticulin at the glomerular root. Reticulin is totally absent between the tubule and vessels in the mouse, cat (Text-fig. 3), and rabbit, and there are multiple gaps in adult man (Text-fig. 4). The condition in the child closely resembles that in the mouse, cat, and rabbit. Thus the cytoplasm of the cells of the macula densa is only separated from that of the cells of the arterioles by the cell-membranes. The absence of any basement membrane at the glomerular root would facilitate exchange between these cells of the tubule and the arteriolar cells, which in this situation possess the specific granules.

THE COMPOSITION OF THE GRANULES IN THE CELLS OF THE ARTERIOLES

Ruyter in 1925 described granules in the juxta-glomerular cells of the afferent arteriole in the mouse. Oberling saw them in man, Okkels in the frog, and
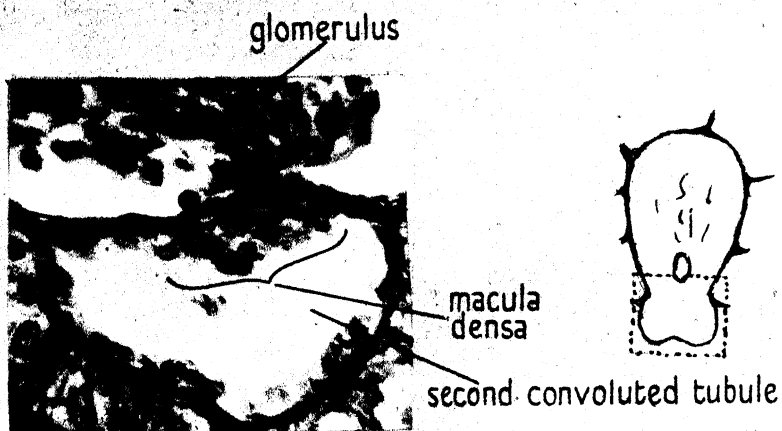
Text-fig. 3. Part of a section of the kidney of a kitten. (Stained for reticulin.)
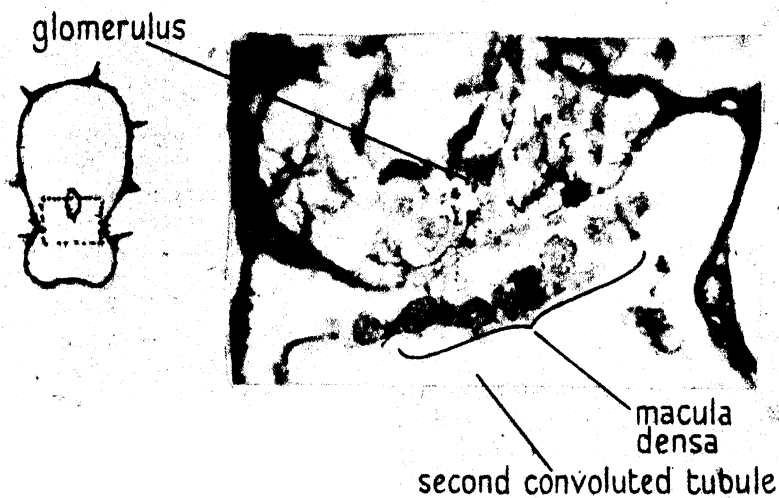
Text-fig. 4. Part of a section of a normal human kidney. (Stained for reticulin.)

Goormaghtigh in the cat and rabbit (references in McManus, 1942). They have been studied by what are essentially mitochondrial methods, which are notoriously capricious. Although a few workers have subsequently recognized and studied them (Kaufmann, Donihue, and Candon), the methods for their demonstration have been unreliable, and there has consequently been delay in the general investigation of them.
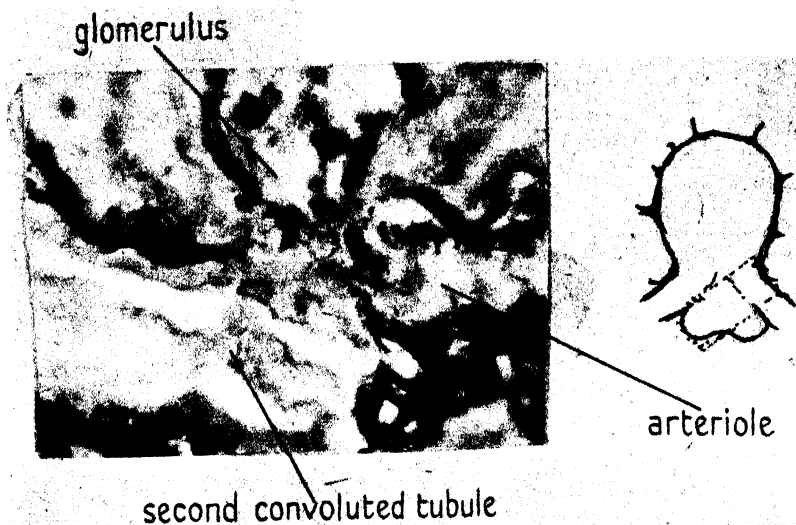
TEXT-FIG. 5. Part of a section of a human kidney affected by malignant hypertension. Note increase in reticulin (? arteriosclerosis), but persistence of gap. (Stained for reticulin.)

The granules are perfectly shown in the mouse by the use of a cobalt-calcium-formalin fixative (McManus, 1946), followed by either sudan black or Masson's trichrome stain. The mitochondria are well preserved and the method seems suitable for a new attack on the kidney. In the rabbit, unlike the mouse, postchroming is necessary and it is advisable to dehydrate in acetone. The same colouring methods are suitable. The human kidneys I have been able to study were not normal but 'crush' cases, in which the granular cells of the arterioles seem to be very numerous. As with the rabbit, post-chroming is necessary but alcohol can be used for dehydration. Once again the granules can be shown with sudan black or Masson's trichrome stain. The similarity of staining reactions suggests that the granules of the juxta-glomerular arteriolar cells of the various species are of similar chemical composition. Colouring by sudan black after fixation in the cobalt fixative and imbedding in paraffin shows that the granules consist (at least in part) of masked lipoid (perhaps lipine).

THE RELATION OF THE GRANULAR CELLS TO THE RENAL TUBULE

The distribution of masked lipoid in the kidney, especially in the mouse, but also in the rabbit and man, as shown by the cobalt-sudan-black technique and by later variants of the same method, presents features that would interest students of the kidney. The clearness with which the granules are shown is striking. The part of the afferent arteriole nearest the glomerulus seems to be composed entirely of these granular cells in the case of the mouse. Hardly less striking is the complete absence of masked lipoid in the macula densa. There is an equally strong contrast in this respect between the macula cells and the adjacent cells of the same tubule, which are rich in masked lipoid.

The facts suggest that something passes between the lumen of the tubule and the cells of the arteriole, and that this movement is accompanied by the movement of lipoids, which aggregate in the arteriolar cells. The direction of flow is suggested by the reversal of the Golgi element in the cells of the macula densa, for in this region—and in this region alone—the Golgi element lies on the side of the nucleus directed towards the base of the cell, that is, towards the side on which there is contact with the granular cells of the arteriole.

In a limited series of cases of chronic nephritis which have already been mentioned (in McManus, 1942), the tubules were not uncommonly found to be separated from the arterioles. I have now somewhat extended the investigation of these and similar cases, especially by studying the distribution of reticulin. The gap in the reticulin at the glomerular root appears often to be obliterated in some cases of chronic glomerulonephritis, but not in the cases of arteriosclerosis or malignant hypertension (Text-fig. 5) which were studied.

SUMMARY

There is a gap in the reticulin at the glomerular root of the mammalian kidney. This gap leaves the macular segment of the second convoluted tubule in intimate contact with the arteriolar cells, which in this region contain granules consisting of or containing masked lipoid. Methods are given for the demonstration of these granules in the mouse, rabbit, and man.

The cells of the macula densa of the mouse are poor in lipoid, and there is thus a striking contrast both with the adjoining cells of the same tubule and with the adjacent arteriolar cells.

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The Action of Diethylstilboestrol on the Prostatic Epithelium of the Mouse

BY

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With one Plate and and Text-figure

CONTENTS

1. Introduction

THE gross effects of oestrogens on the male reproductive system in rodents have been described by numerous authors as involving testicular degeneration, and hypertrophy of the accessory glands and ducts. While the seminal vesicles of rats undergo fibrosis and growth of smooth muscle without any epithelial changes, both the prostate and seminal vesicles in mice show epithelial hyperplasia, followed by metaplasia and keratinization after prolonged treatment. The initial stages of this oestrogenic stimulation of the prostatic epithelium in mice have not been investigated with cytological techniques. Moore, Price, and Gallagher (1930) described characteristic cellular changes in the prostatic epithelium of rats in response to castration and to injection of preparations containing androgens. Consistent morphological variations in the Golgi apparatus of the epithelial cells were found to accompany epithelial regression or hypertrophy. Thus the presence of androgen in a preparation could be tested by injection followed by cytological examination of the prostatic epithelium.

Experiments were undertaken to determine when structural changes may first be detected in the epithelial cells of the various lobes of the mouse prostate following oestrogen administration, and to follow the recovery stages after the treatment was discontinued. The cytological response of the

epithelial cells was investigated in order to determine whether male un-castrated mice might be used in testing for the activity of oestrogen.

2. Material and Methods

Young male mice belonging to an inbred strain (R III) were selected for these experiments. A total of 32 mice, aged 4–5 months, received 10 mg. pellets of the synthetic oestrogen diethylstilboestrol by subcutaneous implantation, according to the technique of Deanesly and Parkes (1937). A number of mice were sacrificed at intervals from 4 to 20 days after treatment. Ten untreated mice were used as control material. In order to study the cytology of the recovery phases of the prostatic epithelium following oestrogen administration the pellets were removed after 20 days' implantation. A number of mice were then killed at periods ranging from 10 to 20 days after oestrogen treatment had ceased.

The anterior, dorsal, and ventral prostatic glands were removed under a binocular dissecting microscope for separate fixation. Preparations of the Golgi apparatus were obtained by treating the tissues by Kolatchev's method as modified by Nassonov. All sections were subsequently stained with Orange G in clove oil. Histological preparations were made from material fixed in Dubosc-Brasil's modification of Bouin's solution and stained by Masson's light green-eosin technique.

3. The Structure of the Normal Prostatic Gland in R III Mice

The nomenclature of the prostatic lobes in rodents has been a subject of much confusion (Rauther, 1903; Disselhorst, 1904; Walker, 1910; Burrows, 1934; Korenchevsky and Dennison, 1935). The several glands, generally referred to as the lobes of the prostate in the mouse, have been described by Deanesly and Parkes (1933) as consisting of paired anterior, ventral, and dorsal lobes, together with a small median gland (see Text-fig. 1A). The last, more frequently termed the ampullary lobe (Fekete, 1941), is a tubular gland lying adjacent to the ampulla in close connexion with the ductus deferens. It opens into the anterior vestibular portion of the ampulla. Examination of this structure in a number of mice belonging to pure line strains showed much variation in size within different strains. In R III mice only a vestigial gland is present.

The paired anterior lobes, called the coagulating gland by some authors (Burrows, 1934; Fekete, 1941), are imbedded in the peritoneal sheath of the seminal vesicles and open into the dorsal wall of the prostatic urethra. The lining epithelium consists of tall columnar cells arranged in characteristic folds. Moore (1939) and others refer to these anterior lobes as the true prostate.

The paired dorsal prostates, which are very much narrower than the anterior lobes, are nevertheless similar in structure and are attached to the dorsal wall of the urethra (see Text-fig. 1B). The columnar epithelium is less folded than in the anterior lobes. The ventral prostate also consists of

bilaterally symmetrical lobes situated on the ventral wall of the urethra at the base of the bladder, and the tubules composing this portion of the prostate differ from those of the anterior and dorsal glands in that they are lined by a low columnar epithelium which is unfolded. The ventral lobes possess several ducts which open into the urethra on its ventral surface.
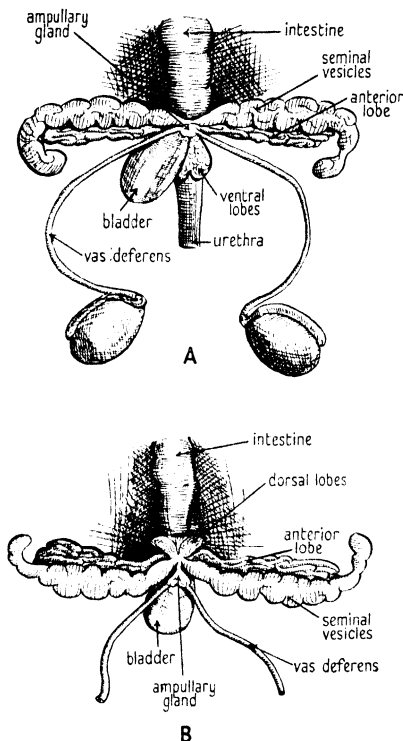
TEXT-FIG 1. Drawings showing ventral and dorsal views of the lobes forming the prostate gland, seminal vesicles, and bladder in a mouse of the R III strain.

4. CYTOLOGY OF THE UNTREATED PROSTATIC EPITHELIUM

Characteristic differences exist between the morphology of the Golgi apparatus in the normal untreated epithelia of the anterior, ventral, and dorsal prostatic lobes. These differences are independent of the normal structural variations occurring in the glandular epithelium during secretory activity (see Pl. 1, figs. 1, 3, 5). Before any significance can be attached to the extensive changes in the Golgi material found after short periods of oestrogenic treatment (see Pl. 1, figs. 2, 4, 6), it is essential to record the cytological appearances of the normal epithelium.

Anterior Prostates. The cells of the columnar epithelium possess a slightly granular cytoplasm and almost spherical nuclei, with the Golgi apparatus

forming a condensed flattened network restricted to the apical region of the cytoplasm. The apparatus in the majority of cells forms an elongated body, orientated transversely to the axis of the cell and situated in close contact with the nuclear membrane (see Pl. 1, fig. 5). Where the mucous membrane is thrown into tall longitudinal folds, projecting far into the lumen, the Golgi substance then becomes compressed and distorted, often losing the appearance of a network and assuming a more compact rounded shape.

Dorsal prostates. The cells of the dorsal prostatic epithelium resemble those of the anterior lobes. The general cytoplasm is, however, more granular and the spherical nuclei stain more deeply. The Golgi apparatus, situated in the apical cytoplasm in close contact with the nucleus, forms a network which is less compact than that found in the anterior lobe cells (see Pl. 1, fig. 3). In the proximal region close to the basement membrane the cells contain secretion droplets which are stained lightly by osmium tetroxide.

Ventral prostates. The low columnar epithelium of the ventral lobe is relatively free from folds. The individual cells are elongated with their oval nuclei confined to the basal region of the cytoplasm (see Pl. 1, fig. 1). The Golgi apparatus forms a characteristic loose network in the apical cytoplasm of these cells and consists of twisted strands, all of which tend to be orientated longitudinally to the axis of the cell (see Pl. 1, fig. 1). The morphology and location of the Golgi material in the epithelial cells of the ventral prostate are therefore quite different as compared with the structure of the apparatus in the anterior and dorsal prostatic epithelia (see Pl. 1, figs. 3, 5).

5. RESPONSE OF THE PROSTATIC EPITHELIUM TO EIGHT DAYS' TREATMENT WITH OESTROGEN

Oestrogenic stimulation does not induce uniform changes throughout all the prostatic lobes. After 8 days' treatment the cytological appearances of the epithelia clearly demonstrate that differences in the degree of sensitivity exist between the three glands (see Pl. 1, figs. 2, 4, 6). There is clear indication that the epithelium of the anterior lobe is more sensitive to stilboestrol than either the epithelium of the dorsal or ventral lobes. The cytological changes which are typical after 8 days' treatment will be briefly described.

Anterior prostatic epithelium. On the eighth day a slight hypertrophy of the whole epithelium, involving an extensive fragmentation and dispersal of the Golgi apparatus throughout the cells, is a consistent feature of this period of treatment (see Pl. 1, fig. 6). The compact Golgi network of the untreated anterior glandular epithelium has entirely disappeared and the enlarged epithelial cells have become filled with fragments of hypertrophied Golgi substance. In most cells there is an aggregation of Golgi material in the apical cytoplasm in the vicinity of the nucleus, and the latter may be distorted. A dispersed Golgi apparatus occupies the entire cytoplasm (see Pl. 1, fig. 6).

Dorsal prostatic epithelium. The cellular changes in the dorsal epithelium are less extensive than those which occur in the anterior epithelium after the

same period of treatment (see Pl. 1, figs. 4 and 6). There is less epithelial hypertrophy and the accompanying changes in the Golgi apparatus are less pronounced. The Golgi network undergoes only a slight fragmentation and occasionally an intact but enlarged Golgi reticulum may be observed in the apical cytoplasm in close relation with the nuclear membrane (see Pl. 1, fig. 4). The characteristic transverse polarity of the apparatus to the axis of the cell, so typical of the epithelium previous to treatment, is lost owing to fragmentation.

Ventral prostatic epithelium. The response of the Golgi apparatus to oestrogen in the ventral epithelium differs markedly from that in the epithelium comprising the dorsal and anterior prostates. After 8 days' treatment a definite but remarkably uniform hypertrophy of the apparatus occurs throughout the whole of the ventral prostatic epithelium. This is not accompanied by any enlargement of the epithelial cells nor by fragmentation or dispersal of the Golgi material. The compact apparatus is localized in the apical cytoplasm, and it still maintains its longitudinal polarity to the cell axis (see Pl. 1, fig. 2).

The difference in response of the Golgi apparatus in the epithelium of the dorsal, anterior, and ventral prostates to treatment can best be appreciated by an examination of Plate 1. The slight reaction of the Golgi material in the ventral epithelium suggests that it is less sensitive to oestrogen than either the anterior or dorsal epithelium.

6. RESPONSE OF THE PROSTATIC EPITHELIUM TO TWENTY DAYS' TREATMENT WITH OESTROGEN

The anterior prostatic epithelium on the twentieth day of treatment has undergone a conspicuous metaplasia. This phenomenon can be macroscopically observed upon laparotomy. At this period the paired anterior glands have become considerably enlarged and the seminal vesicles, which are embedded in the same peritoneal sheath as the anterior lobes, have atrophied. The hypertrophy of the anterior gland is partly due to distension of alveoli by keratinized epithelial debris and accumulation of polymorphonuclear leucocytes. The single layer of cuboidal epithelial cells, still present on the eighth day of treatment, is now replaced by a greatly hypertrophied stratified epithelium. Many of the alveoli are lined by an epithelium 10–15 cells in thickness.

It has been stated by Lacassagne (1933) and Burrows (1934) that the epithelial response of the anterior prostate in rodents to oestrogen provides evidence in support of the contention that these glands are derivatives of the Mullerian ducts. A similar process of rapid cornification of the vaginal epithelium in rodents occurs after short periods of treatment with oestrogens. During keratinization the Golgi bodies in the hypertrophied epithelial cells have undergone changes involving their morphology and distribution. The Golgi material is now present in the form of fine granules irregularly scattered throughout the cytoplasm. Occasionally cytoplasmic structures which stain more deeply than the Golgi material are seen lying adjacent to the enlarged nuclei. These structures, according to Ludford (1924), are nuclear extrusions,

giving rise to keratohyalin granules, and should therefore not be confused with Golgi substance. In most of the greatly enlarged keratinizing epithelial cells the scattered Golgi bodies stain only very faintly until they finally cease to be demonstrated by the Kolatchev technique. Ludford (1925) reports a similar phenomenon occurring in the keratinizing epithelial cells in tar tumours of rodents.

The cytological changes which have taken place within the same period in both the dorsal and ventral prostatic epithelium are less pronounced. The epithelium has undergone little cellular change and no keratinization occurs in these glands, a development entirely confined to the anterior prostate. There is little evidence of cellular proliferation at this stage of treatment, but in the dorsal glands some of the alveoli are now lined by two or three layers of cells. Apart from the increased fragmentation and subsequent dispersal throughout the cytoplasm of the Golgi material, the general cytological appearance of the epithelium is similar to that already described after 8 days' treatment. No desquamated cell debris is seen within the alveoli but numerous leucocytes are present. Proliferation of the ventral glandular epithelium is absent at this stage of treatment. The Golgi apparatus, which is now fragmented, still remains localized in the apical cytoplasm of the epithelial cells, close to the nuclear membrane.

7. The Recovery Phases of the Prostatic Epithelium following Cessation of Oestrogenic Treatment

The keratinized anterior prostatic epithelial cells return to the unstimulated condition 20 days after the removal of the oestrogen pellets. On the twentieth day the keratinized epithelium has disappeared and is replaced by a single layer of cuboidal cells, most of which appear to have resumed their normal secretory functions. Some of the alveoli still remain slightly distended with cell debris and a few epithelial cells may be enlarged owing to the accumulation of secretory products. In the majority of cells the Golgi substance has reformed into a compact but slightly larger network than that seen before treatment. Where the Golgi apparatus has not reformed itself into a network it still remains condensed and localized in the apical cytoplasm. It seems likely that normal secretory activity has not yet occurred in these cells, as it has been shown that oestrogens inhibit the secretory function of the prostate gland (Allen, 1939). The epithelium of the dorsal and ventral glands returns to a normal state more rapidly in accordance with their smaller response to oestrogenic stimulation.

8. Discussion

The present experiments have shown that it is possible to correlate variations in the form and distribution of the Golgi material in the prostatic epithelial cells of the mouse with different periods of treatment with oestrogens, and that the normal morphology of the Golgi apparatus is restored in these cells when treatment is discontinued. It is evident that oestrogenic

stimulation does not cause permanent alteration in the structure of the prostatic epithelium after relatively short periods of treatment, even when keratinization has been induced. The most extensive cytological changes following oestrogen treatment occur in the epithelium of the anterior lobes.

Amongst the more recent work published on the cytological effects of the sex hormones upon cells it should be noted that Moore, Price, and Gallagher (1930) report that the state of the Golgi apparatus in the rat prostatic epithelium has proved to be a reliable indicator for testing preparations for androgenic action. Vazquez-Lopez (1940) has shown that over-stimulation with oestrogen causes an extensive hypertrophy of the Golgi substance in the cells of the islets of Langerhans in the mouse pancreas. Wolf and Brown (1942) have recently described the reactions of the Golgi apparatus in the alpha and beta cells of the pars anterior of the pituitary body following treatment with diethylstilboestrol. In the uterine epithelium of the untreated rat during the oestrus cycle, it has been shown that normal oestrogenic activity is sufficient to produce characteristic cytological changes which are correlated with different phases of the cycle (Horning, 1943).

Before the introduction of the vaginal smear assay for oestrogen by Allen and Doisy (1923), it was generally held that any structural or functional change produced by ovariectomy, which could be restored to normal by injection of ovarian extracts, might be used for the assay of female sex hormone. The microscopic examination of the vaginal smear is regarded as a reliable indicator of the oestrous condition of intact female animals. Rodents, either previously spayed or immature, are employed for the testing of substances having oestrogenic action. This method requires a careful standardization of age and castration methods and has been criticized since it has been found that certain carcinogenic substances (Wright, 1936) are capable of producing the same effects as oestrogens in females. Hechter, Lev, and Soskin (1940) have reported that the alkaloid yohimbine also induces oestrus in rodents. Horning (1943) has suggested that the characteristic changes in the Golgi substance of the uterine epithelium in non-castrated mice, following 8 hours' treatment with diethylstilboestrol, might be used as reliable indicators of the degree of oestrogenic stimulation.

The effects of oestrogens upon male animals have been reviewed by Allen (1939). Golding and Ramirez (1928) were among the first to describe the specific action of oestrogen on immature male rats. They found that this treatment prevented the growth of the testes and their descent into the scrotum. Two weeks after oestrogen administration was discontinued, the treated animals had completely recovered from gonadal inhibition. The production of scrotal hernias in rodents, following relatively short periods of oestrogen treatment, has been reported by several workers (Lacassagne, 1933; Burrows and Kennaway, 1934; Cramer and Horning, 1938). Lacassagne (1936) has further described changes in the epithelium of the bladder in mice following similar treatment, and Gardner (1937) found a striking hypertrophy of the interstitial cells of the testis in response to oestrogens in certain inbred

strains of mice. According to Burrows (1935) oestrogens cause the connective tissue of the ampullary region of the vas deferens in mice to undergo a marked myxomatous transformation after treatment with relatively large doses. He also reported (1937) that hyperplasia of the ducts of the bulbourethral glands occurred after similar treatment.

The present experiments have indicated that it is possible to use male animals, uncastrated and of any age, to test for oestrogenic activity. The cytological reaction in the epithelium of the anterior lobe of the prostate in particular is sufficiently characteristic and precise to serve as an indicator of oestrogen action, being clearly distinguishable from that induced in the same epithelium by the action of androgen. We now have, therefore, cytological criteria with which to assess the activity of oestrogens in the male and female rodent, as well as those originally described by Moore, Price, and Gallagher (1930) for androgens in the male. These methods will prove useful in supplementing the routine methods for the assay of sex hormones and may be employed in the investigation of the varying degrees of reaction to these hormones shown by the many types of epithelial cells and stromal tissues in the reproductive organs.

9. SUMMARY

1. The cellular changes in the prostatic epithelium of an inbred strain of mice (R III), following short periods of treatment with large doses of oestrogen, are described.

2. The Golgi apparatus in the cells of the prostatic epithelium affords a precise indication of the degree of stimulation induced by oestrogenic substances.

3. Specific morphological changes in the Golgi substance occur in the epithelium of the anterior lobe of the prostate after 8 days' treatment. Similar changes, following longer periods of treatment, also occur in the dorsal and ventral lobes.

4. Withdrawal of oestrogenic stimulation after 20 days' treatment with relatively large doses is followed by a return of the prostatic epithelium and the Golgi apparatus to their normal condition in 21 days.

EXPLANATION OF PLATE I

Photomicrographs of the prostatic epithelium of treated and untreated R III mice. All material was impregnated by Nassonov's modification of Kolatchev's method, and subsequently stained with Orange G in clove oil. Magnifications, × 360.

Fig. 1. The prostatic epithelium of the ventral lobe of a control mouse. The columnar epithelium is relatively unfolded, and the Golgi apparatus forms a characteristic loose network in the apical cytoplasm. The Golgi material consists of twisted strands all of which tend to orientate longitudinally to the cell-axis.

Fig. 2. Ventral prostatic epithelium after 8 days' treatment with diethylstilboestrol, showing changes in the Golgi apparatus. It is hypertrophied but retains its compactness and normal polarity.

Fig. 1
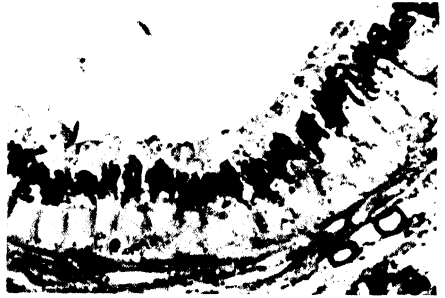
Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 3. Dorsal prostatic epithelium from a control mouse. The Golgi substance forms a compact network in the apical cytoplasm.

Fig. 4. Dorsal prostatic epithelium after 8 days' treatment. The Golgi substance has become dispersed.

Fig. 5. Anterior lobe epithelium from a control mouse, showing the Golgi networks which are confined almost entirely to the apical cytoplasm.

Fig. 6. Anterior lobe epithelium after 8 days' treatment. Note the pronounced reaction of the Golgi substance in response to oestrogen. It has become granular and is dispersed throughout the cytoplasm of the epithelial cells which have undergone hypertrophy.

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The Golgi Material of the Neurones of the Central Nervous System of Sheep Infected with Louping-ill

BY

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With sixteen Text-figures

Introduction

THE histopathology of the nerve-cells of mice and monkeys suffering from louping-ill has been described by Hurst (1931), and an account of the effects of the virus on the neurones of sheep, pigs, and mice has been given by Brownlee and Wilson (1932). The present paper is confined to a description of the changes in the Golgi material of nerve-cells in the brain and spinal cord of sheep infected experimentally with the virus of louping-ill. For comparison the Golgi material of neurones from corresponding regions of the central nervous system of normal animals is described.

Fragmentation of the Golgi material has frequently been observed in degenerating cells and in cells under pathological conditions (Ludford, 1942). The Golgi substance of the epidermal cells of the chick infected with fowl-pox was described by Ludford and Findlay (1926). According to these authors the Golgi material, which in the normal epidermal cell is situated at one pole of the nucleus, becomes hypertrophied during the early stages of infection and in some cases undergoes a reversal of polarity. It breaks up before the virus bodies are fully formed. As the Golgi material is often situated in proximity to the virus bodies, Ludford and Findlay suggest that it may be concerned with the formation or localization of the lipoidal substance deposited around the bodies. Ludford (1928) states that the Golgi substance of the epidermal cells of the cornea of the rabbit infected with vaccinia virus often undergoes hypertrophy, reversal of polarity, and fragmentation. Hypertrophy and fragmentation of the Golgi material is also described by Rio-Hortega (1914) in nerve-cells in a case of paralytic rabies, and by Findlay (1929) in connective tissue cells of rabbits suffering from myxomatosis.

Material and Methods

Small pieces of the cerebrum, cerebellum, medulla oblongata, and the cervical and thoracic regions of the spinal cord of domesticated sheep (*Ovis aries*) suffering from louping-ill were used for the study of the Golgi material. Material from the corresponding regions of the central nervous system of normal sheep was used for comparison. The animals were killed and the tissue

dissected out and placed in the fixing fluid as speedily as possible. The material was prepared according to the methods of Aoyama and Da Fano, and sections were cut at 5 μ and 8 μ in thickness. A number of the sections were toned with gold chloride, and of the untoned sections some were stained with Ehrlich's haematoxylin. The cortex and medulla of the Golgi material was more clearly shown in the untoned than in the toned preparations.

The pathological material was obtained from three sheep infected experimentally with the virus of louping-ill. Sheep I and II, both 6 months old, were killed when showing early symptoms of the disease—un-coordinated gait and occasional spasmodic jerks of the head and limbs. Sheep IV, 9 months old, was in a very advanced stage of louping-ill, lying on the floor, and when raised unable to maintain its balance.

The normal material was obtained from Sheep III, over 1 year old, and Sheep V, 2 months old.

OBSERVATIONS

1. *Cerebrum*

Normal sheep

An examination of the pyramidal cells of the cerebral cortex of the sheep showed that the Golgi material is present as filaments, which vary in length, and as irregularly shaped bodies. In some cells the filaments are very numerous, while in others shorter irregular bodies predominate. The Golgi substance surrounds the nucleus and is usually less abundant at the periphery of the body of the cell. It extends for a considerable distance into the cell processes where long filaments are numerous (Text-fig. 1). In many cases the Golgi material seems to be made up of discrete elements which are often in contact with one another. In other cells, particularly in those of the younger animal (Sheep V), it has the appearance of a definite network. The irregularly shaped bodies consist of an argentophil cortex and an argentophobe medulla which is particularly well shown in untoned sections. As the filaments are slender their division into two regions is less clearly visible, but on careful focusing the argentophobe central part can often be made out.

Sheep infected with the Virus of Louping-ill

Sheep I. The distribution of the Golgi material of the pyramidal cells does not differ greatly from that of the normal tissue, except that in some cells it is greater in amount in the region between the nucleus and the apical dendron. Long filaments are, however, less numerous, and in many cells most, or all, of the Golgi substance is in the form of short irregular bodies (Text-fig. 2). Comparison of these neurones with the cortical cells of the normal animals shows that the Golgi material has undergone hypertrophy, that it rarely

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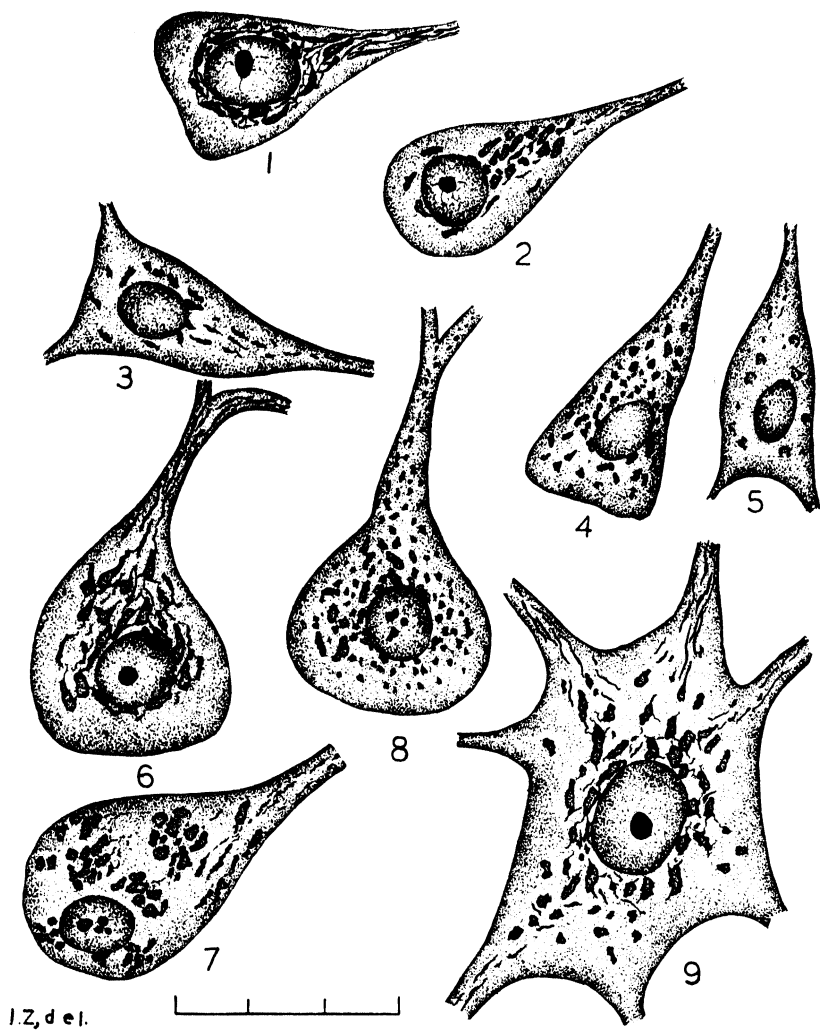
All figures from Aoyama preparations.

FIG. 1. Normal sheep. Pyramidal cell of cerebral cortex. FIG. 2. Sheep I. Louping-ill. Pyramidal cell. FIG. 3. Sheep II. Louping-ill. Pyramidal cells. FIGS. 4 and 5. Louping-ill. Sheep IV. Pyramidal cells. FIG. 6. Normal sheep. Purkinje cell of cerebellum. FIG. 7. Sheep I. Louping-ill. Purkinje cell. FIG. 8. Sheep IV. Louping-ill. Purkinje cell. FIG. 9. Normal sheep. Multipolar cell of medulla oblongata.

The scale represents a length of 30 μ.

assumes the appearance of a definite network, and that long filaments are reduced in number or are absent (Text-figs. 1 and 2).

Sheep II. The location of the Golgi material is similar to that in Sheep I,

except that it is sometimes absent from the cell processes, and is frequently absent or nearly absent from the basal part of the neurone. Filaments, when present, are slender and often appear to be breaking up into smaller bodies (Text-fig. 3).

Sheep IV. According to the form and distribution of the Golgi material the cortical cells are of two types. In one type the Golgi substance is hypertrophied (Text-fig. 4), and in the other it is scanty and in the form of granules and small bodies (Text-fig. 5). In the hypertrophied neurones filaments are few or absent in the cell processes, and when present are always short and granular.

Examination of the pyramidal cells of the infected animals shows that the Golgi material undergoes hypertrophy, and that the filaments become reduced in number. At the same time the Golgi substance is reduced in amount or disappears from the cell processes, and often moves away from the basal part of the cell. Hypertrophy is followed by the fragmentation of the Golgi bodies and by a reduction in the amount of Golgi substance. That all the neurones are not affected equally at the same time is indicated by the presence of hypertrophied cells in the cerebral cortex of Sheep IV. The staining reactions of the cells containing a comparatively small amount of granular Golgi substance indicates that they are necrotic.

2. *Cerebellum*

Normal Sheep

Owing to their large size the Purkinje cells are favourable subjects for investigation. The Golgi material closely surrounds the nucleus and extends laterally towards the periphery and into the basal part of the cell process; it does not extend to the peripheral region in the basal part of the neurone (Text-fig. 6). It consists of long filaments and irregularly shaped bodies, many of which are in contact so as to give the appearance of a network. The filaments are present chiefly in the region anterior to the nucleus, and are all directed towards the cell process into the basal part of which many extend for some distance. The outer deeply argentophil part and the inner argentophobic region are clearly visible in the larger masses of Golgi material.

Sheep infected with the Virus of Louping-ill

Sheep I. Long filaments are less numerous than in normal material, and those present in the cell process often appear to be breaking up to form smaller elements. Irregularly shaped bodies are numerous. In some neurones there is a greater concentration of Golgi substance between the nucleus and the cell process than in those of the normal tissue, and there is often a tendency for several Golgi bodies to lie close together so as to form small groups in the cytoplasm. In many cells the nucleolus is breaking up, and sometimes darkly stained material is present round it (Text-fig. 7).

Sheep II. The Golgi material is similar to that of the Purkinje cells of Sheep I, except that the grouping of several Golgi elements together is less pronounced.

Sheep IV. Golgi material is present as irregular masses. Long filaments are absent and comparatively little Golgi substance is present in the process (Text-fig. 8). In some cells the Golgi bodies are small and are probably formed by fragmentation of larger masses.

It is concluded that the first visible results of infection of the Purkinje cells are hypertrophy of the Golgi material, the disappearance of long filaments, a reduction in the amount of Golgi substance in the cell process, a tendency to a concentration of Golgi material anterior to the nucleus, and for a grouping of Golgi bodies in the cytoplasm. This is followed by fragmentation.

3. *Medulla Oblongata*
Normal Sheep

The following account is based upon the examination of the large multipolar nerve-cells of the medulla oblongata, which contain a large number of filamentous and irregularly shaped Golgi bodies. As in the normal cells of the cerebrum and the cerebellum, many of the bodies seem to be joined to form a network, and filaments extend into the cell processes (Text-fig. 9). In the cells of the medulla of the lamb (Sheep V) the Golgi material is greater in amount than in the older animal; it often more closely surrounds the nucleus and is concentrated in the neighbourhood of one or more of the cell processes (Text-fig. 10). The Golgi elements are similar in appearance to those of the cerebrum and cerebellum.

Sheep infected with the Virus of Louping-ill

Sheep I. In some of the multipolar cells numerous long filaments are present, while in others most of the Golgi substance is in the form of irregular bodies. Filaments are more numerous in the basal part of the cell processes than elsewhere, but in many cases the Golgi material in these regions is considerably less than in the corresponding parts of the normal cells (Text-fig. 11).

Sheep II. Most of the neurones are similar to the cells of Sheep I, but in some cases the large masses of Golgi material appear to be breaking up.

Sheep IV. The Golgi bodies are smaller than in normal cells. Short filaments may be present in the processes but are absent from other parts of the neurones (Text-fig. 12).

The Golgi material of the infected multipolar nerve-cells of the medulla undergoes hypertrophy which is not so marked as in the pyramidal cells of the cerebral cortex and the Purkinje cells of the cerebellum. The chief effects of the virus seem to be the disappearance of long filaments, a reduction in the amount of Golgi substance in the processes, and finally fragmentation of the Golgi bodies.

4. *Spinal Cord*
Normal Sheep

The multipolar motor nerve-cells of the ventral horns were chiefly used for this part of the study. Cells in other parts of the cord were also examined,
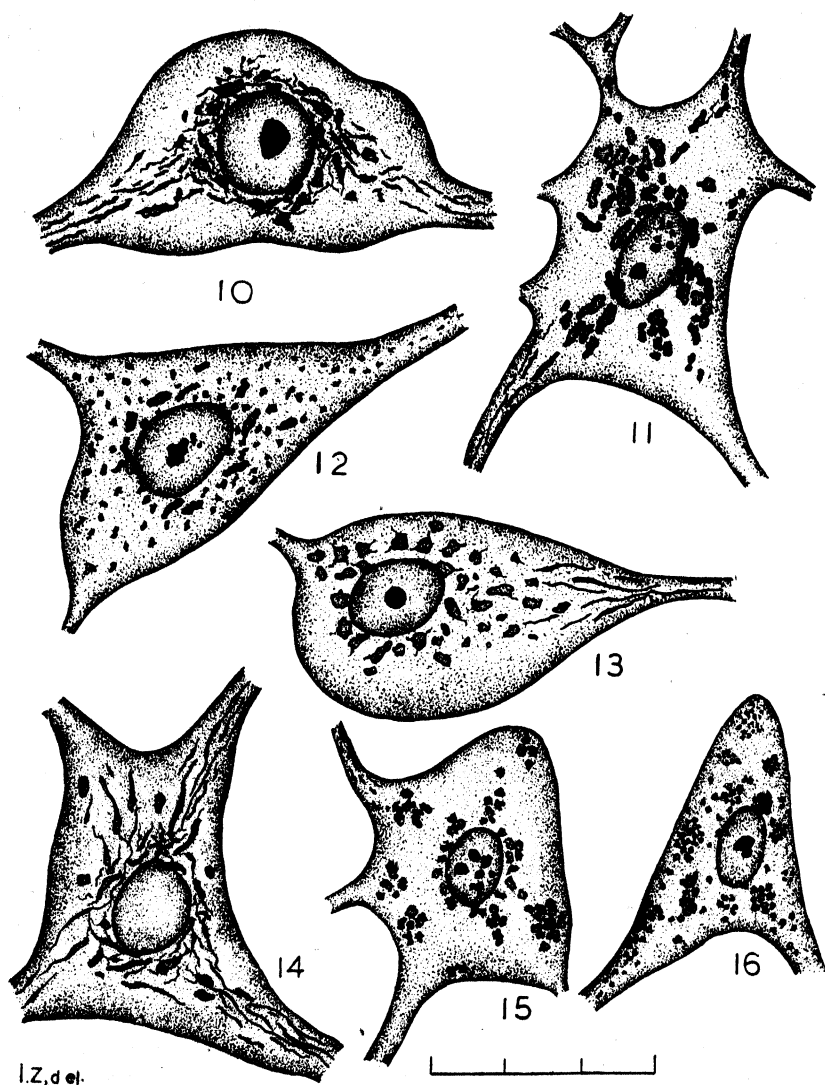
All figures from Aoyama preparations.

FIG. 10. Lamb (Sheep V). Multipolar cell of medulla. FIG. 11. Sheep I. Louping-ill. Multipolar cell of medulla. FIG. 12. Sheep IV. Louping-ill. Multipolar cell of medulla. FIGS. 13 and 14. Normal sheep. Motor nerve-cells of ventral horn of spinal cord. FIG. 15. Sheep I. Louping-ill. Motor nerve-cell of ventral horn of spinal cord. FIG. 16. Sheep IV. Louping-ill. Motor nerve-cell of ventral horn of spinal cord.

The scale represents a length of 30μ.

and the distribution and structure of the Golgi material was found to be similar to that in the cells of the ventral horns.

The form and arrangement of the Golgi material varies somewhat in different cells. In some of the neurones irregularly shaped bodies predominate and in others filaments are very numerous. In a few cases the Golgi substance surrounds the nucleus and appears to form a network. Filaments are present in the basal region of the axon and the dendrons (Text-figs. 13 and 14).

Sheep infected with the Virus of Louping-ill

Sheep I. The Golgi material of some of the motor cells of the ventral horn is hypertrophied and is fairly evenly distributed through the cytoplasm; filaments are not numerous. In a large number of neurones it closely surrounds the nucleus, or forms several groups scattered through the cell. Filaments are short or absent, and the irregularly shaped Golgi bodies are smaller than in normal tissue. The Golgi material is reduced in amount or is absent in the majority of the cell processes. Several small nucleoli are frequently present (Text-fig. 15).

Sheep II. The distribution and structure of the Golgi material is similar to that of Sheep I.

Sheep IV. The Golgi substance of some of the motor nerve-cells is hypertrophied, but in most cases it consists of comparatively small bodies either concentrated about the nucleus or else situated in small groups distributed through the cytoplasm. It is often absent from the axon and the dendrons (Text-fig. 16).

The changes in the morphology and distribution of the Golgi substance within infected motor nerve-cells of the ventral horns is very marked. The Golgi material varies in distribution, but in all the cells examined it consists of bodies which are smaller than those of the normal tissue, and a net-like structure is never present. The first visible change in the Golgi material is hypertrophy, and this is followed by a grouping of the Golgi elements and their fragmentation to form smaller bodies. Infection is also followed by a breaking up of the large filaments of the normal cell, and the reduction and disappearance of the Golgi substance in the axon and in the dendrons.

DISCUSSION

Observations on the neurones of the central nervous system of normal and infected sheep show that the Golgi material undergoes changes consequent upon the invasion of the cells by the virus of louping-ill. In all the types of infected nerve-cells examined by the writers the Golgi substance hypertrophies and finally breaks up. The main visible changes are, therefore, similar to those described by Rio-Hortega (1914), Findlay (1929), Ludford and Findlay (1926), and by Ludford (1928) as due to the invasion of cells by other viruses. In addition certain other features of the behaviour of the Golgi material are noted.

The cells of the spinal cord and the pyramidal cells of the cerebral cortex show the greatest changes. The Golgi material of the Purkinje cells of the cerebellum differs considerably from the normal, but not to the same degree as in the neurones of the cerebrum and spinal cord. The Golgi material of the multipolar cells of the medulla does not undergo marked hypertrophy, but, like that of the other cells investigated, finally breaks up. In the neurones from the different regions the Golgi substance in the cell-processes becomes reduced in amount and in some cases disappears completely. In the cerebellum and spinal cord the Golgi elements often form groups scattered through the cytoplasm. The Golgi material tends to move away from the basal part of infected cells of the cerebrum and cerebellum. Certain cells in the cerebellum, medulla, and spinal cord of the infected animals, particularly in Sheep IV, contain numerous argentophil granules. As some of these cells stain deeply with haematoxylin and others stain very faintly, it was not possible to determine with certainty if they are necrotic or imperfectly preserved by the silver technique. If the cells are necrotic the argentophil granules may represent a later stage of fragmentation of the Golgi material than is described in the preceding sections of this paper.

Hurst (1931) found that in experimental louping-ill of the monkey the Purkinje cells of the cerebellum were severely affected, but that in the mouse necrosis may be slight. Brownlee and Wilson (1932), describing the histopathology of the nervous system, observe that necrotic neurones were not identified in the cerebral cortex of sheep infected experimentally with louping-ill, but were present in two natural cases. In both experimental and natural cases a variable number of Purkinje cells were always destroyed. Necrotic nerve-cells were also observed in the medulla and spinal cord. In the pig there was relatively little destruction of the nerve-cells, while in the mouse the principal lesion was necrosis of most of the large neurones of the medulla and spinal cord. The observation of Brownlee and Wilson that necrotic cells are not present in the cerebral cortex of experimental sheep, but are present in the cerebellum, medulla, and spinal cord, suggests that the cells containing argentophil granules present in the animals described in the present paper are necrotic and in a late stage of the fragmentation of the Golgi material. The writers found that the Golgi substance was not always at the same stage of degeneration in all the cells of a particular region of the brain or spinal cord. This agrees with previous work on the histopathology of louping-ill.

On comparing the Golgi material of the neurones of the brain and spinal cord of the lamb (Sheep V) with similar cells of the normal adult sheep it was found that in the lamb a greater number of cells possessed Golgi substance which appeared to form a network around the nucleus, and which was often composed of very thick strands. Andrew (1939) claims that in the nerve-cells of young mice the Golgi material consists of large oval bodies connected by short threads to form a reticulum. In older animals the bodies connected by threads are smaller, and in some cases threads are absent. In old mice the Golgi material is present as granules scattered through the cytoplasm.

Many cytologists believe that the Golgi material is always present as separate bodies and that the appearance of a network is an artifact (Bourne, 1942). In the nerve-cells of the sheep it is impossible to determine with certainty if a network sometimes exists. The occurrence of separate bodies in a large number of the cells of the normal sheep examined suggests that the Golgi substance is always present as discrete elements which under certain conditions may be situated close together in the cytoplasm.

SUMMARY

1. The Golgi material of the pyramidal cells of the cerebral cortex, the Purkinje cells of the cerebellum, and the multipolar cells of the medulla oblongata and ventral horns of the spinal cord of the sheep is present as filaments and as irregularly shaped bodies. In some of the cells, particularly in the lamb (Sheep V), the Golgi material has the appearance of a network. As it is frequently present as separate bodies it is suggested that it may always consist of discrete Golgi elements which are sometimes situated in close proximity or in contact with one another. Filamentous Golgi elements are present in the basal part of the cell processes.

2. An examination of neurones from the corresponding regions of the central nervous system of sheep infected experimentally with louping-ill showed that the Golgi material undergoes changes consequent upon the invasion of the cells by the virus. The Golgi material undergoes hypertrophy, and at the same time there is a reduction in the number of filamentous Golgi elements and a reduction in the amount of Golgi substance present in the cell processes. These changes are followed by fragmentation. All the neurones of a particular region are not affected equally at the same time. The Golgi material of the Purkinje cells tends to form groups in the cytoplasm prior to fragmentation. In the multipolar cells of the medulla oblongata the hypertrophy of the Golgi material is not as great as in the other regions of the central nervous system. The Golgi material of the motor nerve-cells of the ventral horns of the spinal cord undergoes considerable hypertrophy which is followed by a grouping of the Golgi elements and fragmentation.

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A Quantitative Study of the Osmium Impregnation of the Contractile Vacuole of *Chilomonas paramecium* (Cryptomonadina)

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With two Text-figures

INTRODUCTION

THE application of the metazoan Golgi techniques of Weigl (Mann-Kopsch) and Kolatchev to Protozoa has shown that the region immediately surrounding the contractile vacuole in many organisms is very osmiophilic. This was first shown by Nassonov (1924, 1925), who demonstrated the presence of osmiophil cortices around or associated with the contractile vacuoles of *Paramecium caudatum*, *Lionotus folium*, *Nassula laterita*, *Dogielella* sp., *Childon* sp., *Campanella umbellaria*, *Epistylis gallea*, *Zoothamnium arbuscula*, *Vorticella* sp., and *Chilomonas paramecium*. Nassonov attempted to homologize the osmiophil material and the contractile vacuole in these forms with the Golgi apparatus in metazoan cells—a hypothesis which was only accepted in part by later workers.

The osmium techniques have since been applied to a great many different Protozoa, and a vast amount of literature has been published on the whole question of the homology of the Golgi apparatus in Protozoa; this problem has recently been reviewed in detail (Smyth, 1944) and will not be discussed further here.

Apart from the question of homology of the Golgi apparatus, the fact that there is in many Protozoa a ring of osmiophil material surrounding the contractile vacuole is in itself of interest. There is much evidence from the literature of the problem to show that in the hands of different workers the osmium techniques have produced very different results—even in work on the same organism. Any cytologist who has worked constantly with the osmium technique is aware of the fact that it is very inconsistent in its results, sometimes giving beautiful preparations, at others failing to impregnate completely. Gatenby (1941) writes: 'As regards the technique, it must be at once admitted that it can be capricious. The reason or reasons for this are unknown to cytological technicians.'

It is not surprising then to find, that with work based on such a fallible technique, discrepancies in the results of different workers are common. For example, in the case of the Cryptomonad, *Chilomonas paramecium*, Nassonov (1924) impregnated the contractile vacuole with osmium, but did

not mention whether his preparations were always successful; in the same organism Hall (1930) described the contractile vacuole wall blackened in only 54 per cent. of the specimens; Gatenby and Smyth (1940) stated that in normal undividing cultures of *Chilomonas* the contractile vacuole was impregnated in 99 per cent. of the organisms examined; Patten and Beams, however, were unable to impregnate the contractile vacuole in this organism, though specimens of *Euglena* in the same culture solution were successfully blackened. Similarly, in *Colpidium colpoda* the present writer (1941) showed that the contractile vacuole in this form has a very well-marked osmiophilic cortex, the presence of which had been previously denied by Hall and Alvey (1933) using essentially the same technique.

Numerous other examples could be quoted from the literature of protozoan cytology, but those given above suffice to show that there is a need for an investigation into the factors governing osmium impregnation, and that until these factors are fully understood it seems likely that further confusion will only arise in future work on this problem.

In the present paper a preliminary investigation into some of the possible factors governing impregnation of the contractile vacuole in *Chilomonas paramecium* is described. This organism has the advantage that it has been investigated cytologically by several workers and its general morphology is consequently well known; it is easy to obtain in almost pure cultures which can be maintained for some considerable time without difficulty, and it is sufficiently large to enable its contractile vacuole and related structures to be observed without difficulty.

PREVIOUS WORK

Only one attempt has been made to throw light on the osmic impregnation of Protozoa by a quantitative investigation. MacLennan (1940) investigated the impregnation of the contractile vacuoles of *Actinosphaerium eichhorni*, *Epidinium caudatum*, *Eudiplodinium maggii*, *Haptophrya michiganensis*, *Ichthyophthirius multifiliis*, *Metadinium medium*, and *Ostracodinium monolobum*. Only in *Haptophrya* and *Metadinium* was the contractile vacuole impregnated in 100 per cent. of the organisms examined. In the remaining ciliates the percentage impregnation was considerably lower, varying between 33 and 64 per cent. MacLennan attempted to explain the inconsistency of impregnation of the contractile vacuole in these forms as being due to the fact that the osmiophily varies with the phase of the vacuole. He states that 'the impregnation of the contractile vacuoles is consistent *when like functional stages are compared* and the apparent inconsistency in impregnation shown in Table 1 (MacLennan's paper) is due to lack of analysis of the data'. He claims that the quantitative variation as shown by his results is correlated with the 'cyclic granular aggregations demonstrated in living specimens'. The 'granular aggregations' referred to by MacLennan when concentrated around the contractile vacuole are considered to represent the so-called 'osmiophil cortex' of Protozoa; he considers there is no true osmiophil

vacuolar membrane in any of the forms studied except *Haptophrya*. Although MacLennan states, 'The slides were searched systematically with the aid of a mechanical stage and all the individuals of the species in question were studied and the impregnation recorded', no details of the numbers of organisms studied were given, nor does he give any statistical evidence as to the accuracy of his results.

MATERIAL AND METHODS

An account of the Weigl method as used for Protozoa has already been given in a previous paper (Gatenby and Smyth, 1940), but a slight variation of the usual technique is used in the present work and will be described here in some detail.

The organisms were obtained from an infusion of soil and leaves to which some boiled hay solution was added. Unlike most flagellates common in hay infusions, *Chilomonas paramecium* will remain in large numbers in such a culture—apparently being able to withstand quite large changes in pH. A good culture will remain in a flourishing condition for several months, provided a little fresh tap-water is added to keep up the water-level, and the culture is covered to keep bacterial pollution at a low level. The organisms were concentrated by gentle centrifuging in a hand centrifuge. After concentration the flagellates were shaken up to break up any clumps and were allowed to remain in about $\frac{1}{4}$ in. of solution in the centrifuge tube for half an hour to allow any cytological disturbances caused by centrifuging to subside. Fixation with Mann's fluid followed. In order to keep uniformity throughout the series of experiments, the amount of fixative was kept constant—3 c.c. were used in every case. This was introduced into the centrifuge tube by means of a fine pipette—the fixative being squirted in very suddenly to produce as instantaneous and uniform a fixation as possible. The tube was then shaken further for a few minutes to complete the mixing. After fixation the organisms were washed in two changes of distilled water—15 minutes each—brought into 3 c.c. of pure osmium tetroxide solution, and transferred to an osmication tube and placed in an oven at 32° C. Details of the tubes and the fixation varied in different experiments and will be described under the various sections. After osmication was completed, or, in some experiments, during the process of osmication, the organisms were removed from the osmium tetroxide with a fine pipette and mounted in a drop of Farrants's medium. This latter mounting medium allows of surprisingly clear cytological observation of the osmicated Protozoa, and has the advantage that it enables a very small number of the flagellates to be removed and examined—a procedure which is difficult if they have to be dehydrated, cleared, and mounted in balsam. The percentage of impregnated organisms was counted carefully by observation under oil immersion, the field being moved uniformly by means of a mechanical stage. In each preparation the number of impregnated organisms in the first 500 observed were taken.

MORPHOLOGY OF CHILOMONAS PARAMECIUM

A detailed account of the morphology of this Cryptomonad has already been given in a previous paper (Gatenby and Smyth, 1940) and only a brief description will be included here. The organism is about 20 μ in length, but the size varies considerably with the state of nutrition. The nucleus is spherical and median with a large nucleolus. There are two anterior flagella. The gullet is deep and contains peripheral trichocysts. The single contractile vacuole is anteriorly placed, and lying between it and the nucleus are two (in old cultures) or one (in rapidly dividing cultures) large ovate endoplasmic bodies, which have been identified as pyrenoids. In addition to the osmiophil material associated with the contractile vacuole, a number of small scattered osmiophil granules are invariably found in the region between the vacuole and the pyrenoids.

THE STAGES OF IMPREGNATION

Examination of preparations made at intervals during the osmicating process showed that the impregnation of the contractile vacuole of *Chilomonas* follows a very definite course, which, for the sake of description, can be divided into a number of more or less well-defined stages. The earliest sign of impregnation is the appearance around the contractile vacuole of a ring of light greyish-coloured granules of almost uniform size (A, Text-fig. 1). These granules follow the perimeter of the contractile vacuole very closely, but as far as could be observed do not lie on or in any distinct vacuolar membrane. This ring of granules is complete except for a small break in its periphery, equal in length to about one-sixth of its circumference. The position of this gap is always the same, i.e. it lies on the side of the contractile vacuole nearest to the gullet. The area between the contractile vacuole and the pyrenoids is occupied by a few scattered greyish granules of approximately the same size as those surrounding the cortex.

At a slightly later stage (B, Text-fig. 1) the granules around the vacuole become more heavily impregnated and losing their greyish colour become now a very dense black. At the next stage (C, Text-fig. 1), the material around the vacuole loses its granular nature and appears as a definite osmiophilic ring, which, however, still shows the peripheral break in most cases. Its granular origin is evident from the wavy irregularity of its outline, and the fact that stages intermediate between B and C are easily found in all preparations. As osmication proceeds, the impregnation of the vacuole becomes increasingly heavier and the thin wall found in stage B becomes thickened to form a dense osmiophilic cortex with a small osmiophobic area in its centre (D and E, Text-fig. 1). In preparations osmicated for sufficient time to show both these stages, the peripheral break is seldom visible; there is little doubt from the appearance of the earlier stages that this gap in the cortex becomes obliterated by the over-impregnation of the osmium. Prolonged osmication results in the complete impregnation of the whole contractile vacuole region, and an apparently solid mass of osmiophil material showing an irregular outline is

obtained (F, Text-fig. 1). The impregnation of the central (osmiophobe in earlier stages) area of the vacuole varies quite considerably in this final stage, and is seldom actually as heavy as the cortical region, though this appears to be the case at first sight. By using a very strong and concentrated source of illumination, this central region in some cases can be seen to be made up of a thin osmiophil layer which is in the nature of a membrane lying *within* the more heavily osmiophilic outer layer. In other cases, however, the central region appears to be as heavily impregnated as the outer cortex, and is equally opaque even with very intense light.
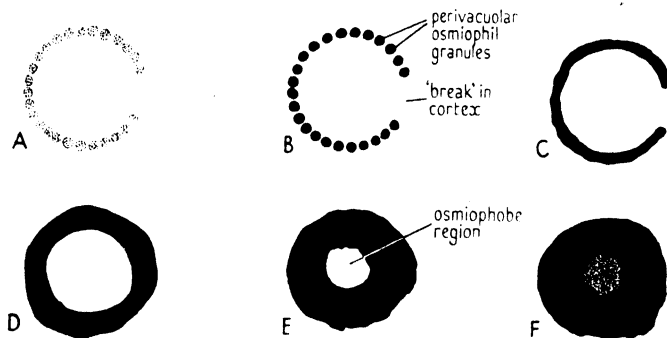
TEXT-FIG. 1. Stages in the impregnation of the osmiophil cortex of *Chilomonas paramecium.* Somewhat diagrammatic.

Although the course of impregnation is relatively easy to follow and interpret, it must be emphasized that the various stages outlined above by no means complete the picture, for since the process of impregnation is a continuous one, as is to be expected, a number of stages intermediate between those described above are found in all preparations. Moreover, the degree of impregnation in any culture after a given time of incubation is not by any means uniform, and a number of different stages can be seen in any one preparation.

An analysis of the percentage of different stages found in a typical culture (using sample A OsO_4) showing 70 per cent. maximum impregnation provides some interesting results. After 16 hours' incubation the first five stages A to E are all visible; about 64 per cent. show stages B or C and only some 17 per cent. are in the earliest impregnation phase. Approximately 16 per cent. have progressed to stage D, and a very small number (3 per cent.) show stage E. The complete impregnation of the vacuole (stage F) is never shown in organisms osmicated for less than 30 hours. After 27 hours' incubation the distribution of the stages follows the course expected, namely, there is a decrease in A, B, and C, which is compensated by a marked increase in D and E. After 66 hours stage E reaches its peak and large numbers have reached the total impregnation stage F, while the earlier stages A to D are much less frequent. Further osmication shows that numbers of organisms previously

at stage E have been converted to F, and in the final observation (162 hours) two-thirds of the impregnated organisms are at the F stage, 21 per cent. are at stage E, and only a very small proportion of the earlier stages (total 12 per cent.) can still be found.

At all stages of impregnation the scattered osmiophil granules—the first appearance of which was noted in the earliest stage—were present. Apart from becoming more densely blackened, their appearance changed little during the later period of impregnation.

IMPREGNATION UNDER NORMAL CONDITIONS

Normal conditions are those described under 'Methods' in the earlier sections. The first set of experiments were designed to determine whether under uniform conditions of fixation and osmication the percentage of organisms that are impregnated is constant.

The tubes used to carry out the osmication in this series of experiments were of ordinary drawn glass with measurements of 5×1 cm. Before use they were carefully cleaned first with soap and water, secondly with chromic acid, and finally rinsed in tap-water followed by distilled water. Fixation time was kept constant at 60 minutes. In one series of experiments a number of organisms were fixed simultaneously in the same centrifuge tube, and, after washing, were divided into three parts and transferred to three separate tubes for osmication. A second series of organisms was treated in *separate* centrifuge tubes, and after washing transferred to three separate tubes. Two samples of osmium tetroxide, A and C, obtained from different manufacturers, were used. In previous cytological studies on Protozoa, A had been found to give good impregnation results and C poor results, but the samples had not been tested quantitatively.

The results of a series of experiments of this nature are given in Table 1. The differences between the figures of the percentage osmication with the two samples of osmium tetroxide were in all cases highly significant, the impregnation with A being nearly twice that obtained with C. Organisms with common fixation and separate osmication showed a high degree of uniformity of impregnation, with standard deviations of 0·85 and 1·22 for A and 2·35 and 0·47 for C. It must be noted, however, that although the impregnation of any group of three tubes with a common fixation was fairly uniform, the difference in the figures for any two experiments of the same type with the same sample of OsO_4 was significant, i.e. the results of any one experiment were not reproducible. Cultures, both fixed and osmicated separately, showed standard deviations between the three tubes—in three out of the four experiments—that were significantly higher than cultures with a common fixation.

In order to investigate more fully the process of impregnation under normal conditions the rate of impregnation of vacuole of two cultures using the two samples of osmium tetroxide was determined. The procedure adopted was identical with that described previously for investigating the

stages of impregnation—namely, samples were taken at intervals during the osmication process and the percentage of impregnated organisms—in this case independently of their stages—were counted.

TABLE I. *Percentage impregnation after common and separate fixation.*
Two different samples of osmium tetroxide used for osmication

OsO$_4$ sample	Fixation	Per cent. impregnation	Mean	Standard deviation	Standard error
A	Common	84·0 82·5 84·0	83·67	0·85	0·49
A	Common	71·0 70·5 73·0	71·5	1·22	0·71
C	Common	30·0 25·0 25·0	26·66	2·35	1·36
C	Common	31·0 31·0 30·0	30·66	0·47	0·27
A	Separate	70·0 75·0 85·0	76·66	6·24	3·60
A	Separate	74·0 70·0 62·0	68·66	5·46	3·15
C	Separate	35·0 30·0 30·0	31·66	2·36	1·36
C	Separate	30·0 33·0 36·0	33·0	2·45	1·42

The results are shown graphically in Text-fig. 2. With sample A the maximum impregnation is almost reached within 16 hours, i.e. the majority of vacuoles which will be finally impregnated become impregnated within this time. With the poorer sample of osmium tetroxide—sample C—the initial impregnation rate is slow, but the percentage impregnation rises slowly with time, to settle to a steady figure in about the same time (approx. 100 hours) as sample A. From these curves it is at once evident that prolonged osmication cannot increase the percentage of impregnated organisms in any single culture.

EFFECT OF MIXING ON IMPREGNATION

During the course of examination and counting large numbers of preparations of osmicated *Chilomonas*, it was noted that in the case of very poorly impregnated cultures, i.e. cultures showing less than 30 per cent. impregnation, the impregnated organisms appeared sometimes in clumps in the field

of the microscope. This suggested the possibility that fixation or osmication, or both, was not uniform throughout the culture solution, and that some parts had been better fixed than others or were in closer contact with the osmium tetroxide solution during the incubation.
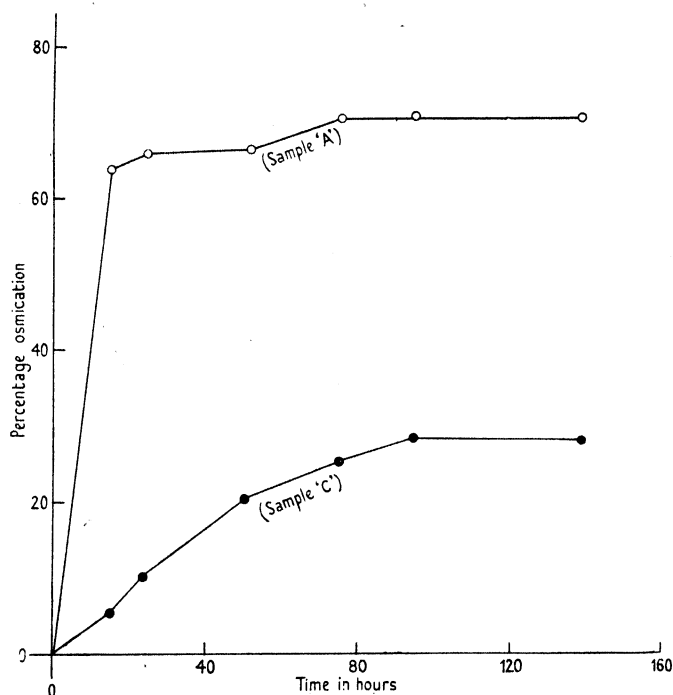
TEXT-FIG. 2. Rate of impregnation of the osmiophil cortex of *Chilomonas paramecium* with two samples of osmium tetroxide.

In order to eliminate any possible error due to this cause, experiments were carried out in which the solutions were kept in constant motion during the entire processes of fixation and osmication. The tubes containing the organisms were placed in a small box fitted to the axle of an electric motor, which rotated five times a second—a speed sufficient to mix the contents thoroughly and yet not centrifuge them to one end of the tube. The organisms were rotated during fixation, washing, and osmication—in the latter case the whole arrangement being fitted into the incubation oven.

The results obtained are shown in Table 2. The percentage impregnation showed a much greater variation with both specimens of osmium tetroxide than when osmicated under normal conditions. This was especially noticeable with sample A, when the impregnation figures in three experiments fell well below 30 per cent.—a low level never obtained in normal experiments with

this sample. In the first set of experiments of this nature ordinary corked tubes were used as before, and it was noted that since the corks came into contact with the osmium solution more frequently as a result of rotation, the former became more heavily blackened than was usual, and it was thought that this might possibly have some effect on the impregnation. In order to eliminate any factor introduced by this the experiments were repeated with tubes that had been sealed completely by means of a fine hot gas flame. Although some of the individual results were higher than those obtained with

TABLE 2. *Percentage impregnation after continual mixing during fixation and osmication, using corked and sealed tubes, with two specimens of osmium tetroxide*

	OsO$_4$ 'A'		OsO$_4$ 'C'	
	Corked tubes	*Sealed tubes*	*Corked tubes*	*Sealed tubes*
	78·0	41·0	40·0	4·0
	61·0	38·0	29·0	30·5
	25·0	85·0	30·0	26·5
	93·0	90·0	17·5	34·0
	20·0	17·0	15·5	16·0
	17·5	21·0	23·0	70·0
Standard deviation	30·0	25·4	8·3	20·4

corked tubes, the variation between the percentage impregnation for a given specimen of osmium tetroxide was still considerable and in no way compared with the more uniform results obtained by using stationary tubes. It is interesting to note, however, that in one case of osmication with c, the effect of rotation gave an impregnation figure of 70 per cent.—a result considerably higher than any previously obtained with this sample in stationary tubes, and comparable to that obtained with the better sample of the osmium tetroxide.

EFFECT OF FIXATION TIME ON IMPREGNATION

Organisms were treated as for normal osmication, but fixed for different periods of 5, 30, 60, and 90 minutes, both samples of osmium tetroxide being used.

Results of the four experiments carried out are shown in Table 3. It is at once evident that with either sample of the osmium tetroxide the impregnation shows no correlation with the fixation time, and that the variation between the impregnations obtained with either A or C for different fixation times is only that approximately to be expected under normal conditions as shown in Table 1, and as far as can be determined the period of fixation—within the limits of the above experiments—has no effect on the final impregnation.

TABLE 3.　*Percentage impregnation for different fixation times, using two specimens of osmium tetroxide for osmication*

Fixation time in minutes	OsO_4 'A'		OsO_4 'C'	
	Exp. 1.1	*Exp. 1.2*	*Exp. 1.3*	*Exp. 1.4*
5	70·0	76·5	33·0	30·5
15	71·0	73·0	40·0	30·0
30	80·0	66·0	31·0	30·0
60	85·0	70·5	31·5	36·0
90	83·0	63·0	36·0	34·0

DISCUSSION

The cytological pictures presented by the contractile vacuole of *Chilomonas* during the various stages of its impregnation are difficult to interpret. The appearance of the osmium precipitate in the form of granules suggests that the so-called osmiophil cortex may be granular in origin. The occurrence of perivacuolar osmiophil 'granules' has been noted previously in a few other Protozoa. In the *Ophryoscolecidiae* MacLennan (1933) has claimed that the warm method of impregnation produced a thick osmiophil membrane around the contractile vacuole, whereas the longer method at room temperature demonstrated the same region to be granular. He also suggested that since the solid cortices around the contractile vacuoles of forms such as *Chilodon* and *Dogielella*, as figured by Nassonov (1924, 1925), showed a distinct granular roughening in the outer region, the solid structures described by him are really artifacts produced by over-osmication.

In *Plagiopyla* (Smyth, 1941) the impregnation of the contractile vacuole goes through a series of stages almost identical with those described in *Chilomonas*: 'The osmiophil cortex first makes its appearance as a thin ring of black beads. On further impregnation the beads coalesce, and the typical osmiophil cortex is produced. Further impregnation makes the ring so thick that the whole structure appears solid.' The phases in this form were not investigated in any great detail, and no figures are available. In *Lagenophrys* (quoted by Gatenby, 1941) Willis has described 'blackening of the accumulated granules which give the appearance of a distinctive Golgi cortex to the contractile vacuole'. Gatenby (1941) has shown that in *Vorticella* during binary fission the osmiophil cortex of the parent cell passes over completely to one daughter organism, whereas a new one is formed in the other by the accumulation of scattered osmiophil granules around the contractile vacuole.

On the other hand, it must be emphasized that there is some evidence from metazoan cytology that granular precipitates of heavy metals—notably silver—may not necessarily indicate the presence of pre-formed granules. Barnett and Fisher (1943), using the acid silver nitrate method to demonstrate the presence of vitamin C in artificial mixtures of olive oil, ground glass, or kieselguhr in gelatine solutions, found that the form of the silver precipitate

had 'no bearing on the prior localisation of ascorbic acid' and concluded that 'it is unjustifiable to infer the whereabouts of ascorbic acid within the cell from the site of the silver precipitates by the silver nitrate method'. Bourne (1944), however, has criticized their results and has cited cases where exact correspondence was obtained between granular mitochondria stained in Janus green B and vitamin C granules in adjacent frozen sections of various tissues (Bourne, 1935; Leblond, 1934; Giroud, 1938). Thus, in some cases at least, metallic precipitates in the form of granules would seem to indicate the presence of pre-formed granules. Nevertheless, the perivacuolar 'granules' in *Chilomonas* have never been demonstrated by methods other than those based on the reduction of osmium tetroxide, and the possibility that these osmiophil granules merely represent the first stage in the reduction of the osmium tetroxide in a specialized area, and not necessarily pre-formed granules, must therefore not be overlooked.

Nassonov (1924) figures the 'broken-ring' type of osmiophil cortex in *Chilomonas* as representing that of a contractile vacuole in diastole, and considered the completely impregnated vacuole to represent the condition found at *systole* by the collapsing of the osmiophil cortex. From the fact that in the present experiments the very early impregnation stages (about 16 hours) showed only open rings, whereas the very prolonged stages showed mainly completely impregnated vacuolar areas, it must be concluded that the so-called 'systole' condition of Nassonov is simply due to over-impregnation, and that the osmiophil material in *Chilomonas* does not change during the vacuolar cycle.

Since the contractile vacuole itself undergoes collapse, whereas the osmiophil cortex does not, it is evident that the latter lies *outside* the region of the contractile vacuole proper. Some workers have taken the impregnation of the vacuolar region as being indicative of the presence of a vacuolar membrane, but if the osmiophilic area lies *outside* the vacuole, this provides no evidence for the existence of such a membrane.

The presence of a 'break' in the osmiophil ring in the early stages of impregnation suggests the presence of a permanent pore by means of which the contractile vacuole is enabled to discharge its contents to the exterior. According to MacLennan (1944), a permanent pore exists in the contractile vacuole region of the *Ophryoscolecidae* where a similar 'break' is found, whereas in *Amoeba proteus*—where there is no perivacuolar osmiophil material (Singh, 1938)—the vacuolar pore is lacking.

The results of the quantitative experiments on the degree of impregnation show that the percentage impregnation with the same specimen of osmium tetroxide and the same fixing fluid can vary quite considerably even under the most carefully controlled conditions. The fact that organisms fixed in the same tube and osmicated separately give much more uniform results than organisms fixed separately, indicates that there is some controlling factor introduced *at the time of fixation*. The only materials concerned in the fixation process are (*a*) the culture solution containing the organisms; (*b*) the fixing

fluid; (c) the centrifuge tube. Now since both (a) and (c) are identical in any series of experiments, the factor must be introduced by either the centrifuge tube itself or by the actual physical action of fixation. Each centrifuge tube used in the present experiments was approximately the same size and shape and had been treated by a similar cleaning process; it is difficult to imagine that there could be any difference between the tubes sufficient to cause such variation as is seen with specimen A (Table 1) where the percentage impregnation varied from a minimum of 62 per cent. to a maximum of 85 per cent.—a difference of 23 per cent.! It is always possible, however, that the process of impregnation is so delicate that the slightest trace of some impurity may upset it, as it is well known that in many chemical and physical reactions a trace of some impurity can act as a retarding agent which inhibits the normal working of the process (Bailey, 1937). It is always evident that the actual process of fixation can never be *identical* since a personal factor is always introduced, and it is possible that the speed and uniformity of the introduction of the fixative into the centrifuge tube plays a part in the determination of the maximum percentage impregnation. Since the controlling factor is introduced at the time of fixation, there is no evidence to suggest that with a given specimen of osmium tetroxide the later stages in the osmication play any part in the determination of the final impregnation figure. The fact that different specimens of osmium tetroxide give such widely different figures is further evidence that the impregnation process itself is a very delicate one that is easily inhibited by impurities; indeed it is difficult to account for the results of different samples by any other hypothesis. There is no evidence to indicate the nature of these impurities, but it is well known that the pH is an important factor in the reduction of metals from solutions, and it is possible that the impurities in different samples are such as to affect this factor.

The results of experiments with the solutions in constant rotation during fixation and osmication indicate that there is nothing to be gained by this technique which only produces extremely variable results. In some individual cases these are higher than those of normal fixation, but on the whole give very poor impregnation. It is difficult to account for the irregular results produced by the mixing, and there is no evidence to indicate what factor is thereby introduced. Considering the small size of *Chilomonas paramecium*, it is not surprising to find that the length of fixation time has no effect in determining the impregnation percentage; but it is interesting to note that the power of impregnation with a poor specimen of the osmium tetroxide is not increased by prolonging the fixation time, as was first thought might be the case.

MacLennan (1940), from work on a number of Protozoa, stated that the irregularity of impregnation was due to a variation in the osmiophily of the vacuolar region with the phase of the contractile vacuole, and that 'impregnation is consistent, *when like functional stages are compared*' (the italics are MacLennan's). Thus he considered his results to be uniform, and correlated

his findings with the fact that, as opposed to metazoan tissues, we are dealing with organisms so small that each part is in close touch with the fluids concerned in the technique. He writes:

'In the Protozoa, the individual cells are separate and the distance of any granule from the free surface is measured in microns rather than millimeters. Thus, fixation, washing, impregnation and every other stage of the Golgi techniques are uniform with respect to every protozoan in the lot, rather than uniform only with respect to a narrow layer of cells equi-distant from the surface. Because of these advantages which are inherent in the Protozoa, it is possible to achieve uniformity of conditions both with respect to conditions of fixation and impregnation and also with respect to conditions within the cell. With these uniform conditions the Golgi impregnations give uniform results.'

These conclusions are open to serious criticism on the grounds that, as far as can be inferred from the data given in his paper, they are based on the results of a *single* series of experiments. Moreover, he does not take into account irregularities which may arise due to the specimen of osmium tetroxide used. It has been shown in *Chilomonas* that this is a factor of major importance, and it is unlikely that with the Protozoa used by MacLennan the same fact does not apply. It seems more likely that the irregularity of his results is due either to the osmium tetroxide used or to some inhibitory agent rather than to a variation in the osmiophily during the vacuolar phases. It is impossible in *Chilomonas* that the osmiophily varies with the vacuolar phases, for although in the present experiments the maximum impregnation reached with normal osmication was only 85 per cent., in a previous paper (Gatenby and Smyth, 1940)—where a better sample of osmium tetroxide was available—consistent impregnations of 99 per cent. were obtained. Since the vacuolar phase must have varied greatly in the large number of organisms used it is evident that in *Chilomonas* there is no relation between the percentage impregnation and the vacuolar phase.

SUMMARY

1. Examination of the contractile vacuole of *Chilomonas paramecium* during progressive impregnation by the Weigl osmic technique revealed that the so-called 'osmiophil cortex' appeared first as a number of perivacuolar osmiophilic granules. Prolonged impregnation caused these to fuse to form a closed ring which, after very prolonged incubation, became a solid osmiophilic mass.

2. Cultures fixed together and osmicated in separate tubes gave more consistent impregnations, in any one series, than those both fixed and osmicated separately.

3. With the two samples of osmium tetroxide used, after normal fixation, A consistently gave impregnations of 62–85 per cent., and C impregnations of 25–36 per cent.

4. Constant mixing during fixation and osmication was not advantageous, and gave more irregular results than normal methods.

5. Time of fixation, between the limits of 5 to 90 minutes, had no effect on the impregnation.

6. It was suggested that the irregularities of impregnation with a given sample of osmium tetroxide were due to the presence of a trace of some retarding agent, possibly introduced at the time of fixation.

7. It was shown that the osmiophily of the perivacuolar region in *Chilomonas* did not vary with the vacuolar phase.

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On the Structure, Division, and Systematic Position of *Trichomonas vaginalis* Donné, with a Note on its Methods of Feeding

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With one Plate

Contents

I. Introduction

*T*RICHOMONAS VAGINALIS, parasitic in the human vagina, is the type species of its genus. It is the largest, commonest, and most easily observed of the human trichomonads, and may be easily cultivated in media in ordinary use. Further, it is probably responsible, it now seems, for the vaginitis with which it has long been associated (Trussell, 1940; Trussell and Plass, 1940; Hogue, 1943). Obviously, *T. vaginalis* is an organism of some importance to the protozoologist and the physician; yet its life-history is imperfectly known and its structure and systematic position are still in dispute.

In 1926 Wenyon suggested that the characters then believed to separate the trichomonad of the human vagina from *Trichomonas hominis* Davaine, of the human intestine, might be due to the differences in their habitats. Were this so, and *T. vaginalis* and *T. hominis* synonyms, there was an evident possibility that infection of the vagina might be initiated by the migration of flagellates along the perineum from the anus. In the last 20 years, both

experimental and morphological studies of the problem have led to disagreement. The examination of abundant material of *T. vaginalis* has convinced me that this is a good species, separable from all other human flagellates on purely morphological grounds. It is the main purpose of this paper to describe the evidence for this contention.

Andrews (1929) found that *T. vaginalis* in the vaginal secretion was a larger flagellate than *T. hominis* and had a shorter undulating membrane, the axoneme of which was not continued posteriorly into a free flagellum (Fig. 1); *T. hominis* had a long membrane with a free posterior flagellum. But these differences disappeared in culture and *T. vaginalis* assumed the form and dimensions of *T. hominis*. At that time the existing accounts of the structure of *T. vaginalis* by Künstler (1884), Blockmann (1884), Bensen (1910), Reuling (1921), Hegner (1925), and Schmid and Kamniker (1926) had left so much in doubt that Andrews's discovery was not sufficient ground for uniting the species and she did not formally propose to do so. But in 1934 Dobell isolated in culture a strain of *Trichomonas* from the gut of *Macacus nemestrinus*, passed it through the gut of *M. rhesus*, infected a human intestine with it, and, recovering it from the faeces, infected both the vagina and the gut of *M. sinicus*. Meanwhile, Bishop (1931) had given a clear account of the structure and division of *T. hominis* and Dobell referred to his flagellates as indistinguishable from those described by Bishop. He provisionally concluded that the intestinal and vaginal trichomonads *of man* belonged to the same species. This was disputed by Westphal (1935) and Powell (1936), who maintained that both forms were always distinguishable in culture. Karnaky (1934) claimed, on very slight evidence, to have infected the human vagina with intestinal trichomonads, but Stabler, Feo, and Rakoff (1941), Feo, Rakoff, and Stabler (1941), and Stabler and Feo (1941, 1942) failed to do so. Since 1926 new descriptions of *T. vaginalis* have been published by Wenrich (1931, 1939), Bland, Wenrich, and Goldstein (1931), and by Powell (1936). They all considered *T. vaginalis* distinct from any intestinal flagellate, but their argument was marred by disagreements about characters so fundamental in systematics as the structure of the nucleus and many cytoplasmic organs. (The American protozoologists in general recognize various subdivisions of *T. hominis* not universally accepted. This taxonomic treatment makes no difference to the position of *T. vaginalis*, but is based on distinctions which have never been urged for or against uniting *T. vaginalis* with any intestinal flagellates. Throughout this paper *T. hominis* is used in its wide sense to include the intestinal trichomonads of man and some monkeys (see Dobell, 1934).)

Clearly, we cannot assess the systematic position of *T. vaginalis* until the contradictory statements about its structure are resolved and we know enough about its mode of division to support comparison with that of other trichomonads. The following studies have been made to these ends. In addition, plentiful opportunities to observe the living parasites have enabled me to incorporate in this report some notes on their feeding methods.

II. MATERIAL AND METHODS

1. *Material*

All the flagellates used in this work were obtained from patients at the Exeter Women's Welfare Clinic. A little of the exudate was scooped from the vagina in a small spoon, a drop of it diluted with Ringer or the liquid component of the culture medium, and examined for parasites. Material was often available for study within a few minutes of leaving the host, but this is unimportant. Some strains remained active and normal for several hours when left at room temperature and I was never able to observe changes in any flagellate so kept for half an hour; material was almost always examined more promptly than this.

2. *Culture Methods*

I have attempted no systematic investigation of the reactions of *T. vaginalis* to different culture media, but have been concerned merely to find a method by which healthy stocks of flagellates could be maintained for use in the laboratory.

All cultures have been grown on variants of Boeck and Drbohlav's media as described by Dobell and Laidlaw (1926). After preliminary trials it was found that the medium known as Ere (coagulated egg slopes covered with diluted egg albumen) was easy to prepare and keep, and, after modification as described below, gave excellent cultures. Andrews (1929) found it unsatisfactory as excessive bacterial growth necessitated daily sub-culture; this may be remedied by the addition of a few drops of acriflavine (1 : 1000) to tubes containing 4–5 c.c. of liquid.

For some strains at least, additional carbohydrate is not essential for maintaining cultures, though it very greatly improves them. Without it growth may be good for a short time, but the flagellates eventually become extremely small, sluggish, distorted, and few; they recover a little on sub-culture but degenerate again within 24 hours. I have kept some strains in this condition for over 2 months, by sub-culturing every other day. The effect of adding carbohydrate to such stocks was startling. Trussell and Johnson (1941) noted great increases in the populations of cultures after adding glucose, maltose, soluble starch, dextrin, or glycogen to their medium, but, with Ere, this is not the only effect. After passing a strain through 25 sub-cultures in 60 days in ordinary Ere, I transferred it to Ere plus 0·25 per cent. dextrose (= Ere+d). (The substitution of soluble for solid carbohydrate reverts to the original recommendation of Boeck and Drbohlav, 1925.) At the time, prolonged search of three dippings from the old medium revealed two tiny, distorted flagellates, feebly beating their flagella from time to time; the strain had been in this condition for about 6 weeks. After 2 days on the new medium, a glance down the low power of the microscope showed in the first drop examined scores of large, vigorous, and normal trichomonads all over the field. In the old medium, measurements made

2421.1

over the 18 days before the transfer showed a few flagellates 8–9 μ, but most 5–6 μ in length, excluding the axostyle. After 24 hours incubation in the new medium, the first 25 brought at random under the $\frac{1}{12}$ in. objective varied from 10 to 18 μ, and after 48 hours all sizes from 8 to 24 μ, with a mean of 14 μ, were found in 50 measurements. The small individuals were presumably the products of the very rapid rate of division. Over the next 2 months on the new medium, tubes usually lasted for 6–7 days or even, though exceptionally, for a fortnight without sub-culturing.

The addition of sterile rice starch to culture tubes may have a similar invigorating effect, but it suffers from some disadvantages. Starch is difficult to sterilize. The grains, if ingested, tend to distort the bodies of the trichomonads. Some strains do not ingest starch grains at all; but it is of the greatest importance (see below) to understand that such strains may benefit considerably from the addition of starch to their medium; they increase in size, numbers, and mobility, though not to the extent of those fed on dextrose. One fact, important in diagnosis, remains to be noted. According to Dobell (1934), who cultivated *T. hominis* on all varieties of Boeck and Drbohlav's medium, primary cultures from faeces almost always showed abundant growths after 24 hours. *T. vaginalis* is less reliable. Primary cultures, with or without additional carbohydrate, were often feeble and short-lived. Flourishing sub-cultures have been obtained from primaries in which no trichomonads could be found except by prolonged searches of three or four dippings, i.e. from cultures very likely to be passed as negative when examined by quick routine methods. Early sub-culture from the primary tube is essential if negative results are to be accepted.

To summarize, the following method is recommended to give active, healthy, and prolific cultures of *T. vaginalis*. Slopes of whole egg are autoclaved and covered with 4–5 c.c. of a liquid consisting of the whites of 2–4 eggs in a litre of Ringer's solution plus 2·5 gm. of dextrose, sterilized by filtration. Incubate overnight to test for sterility; the medium is then approximately neutral. Inoculate with several globules of vaginal exudate held in a sterile platinum loop and incubate at 37°. Sub-culture next day and thereafter every 4 or 5 days according to the state of the cultures. If bacterial growth becomes excessive, add 3 or 4 drops of acriflavine to each tube for several generations. Trichomonads are found in the whitish pulp at the bottoms of the tubes where the solid component of the medium is breaking down. Sub-cultures are made by transferring from 0·5 to 1·0 c.c. of this in a sterile pipette—not a loop scraping from the slope—to a new tube.

3. *Cytological Methods*

Most authors agree that *T. vaginalis* is difficult to fix and stain satisfactorily, and I have found the reagents commonly employed to preserve cytological detail far more useful than the ordinary protozoological fixatives. Bouin's fluid caused so much distortion of the cytoplasm that it was almost useless. Alcoholic modifications of Bouin were very little, if any, improvement on

the original aqueous formula, but Schaudinn's fluid with acetic acid and Dobell's modification of Zenker's fluid (Dobell, 1943) gave better results. The vapour of osmium tetroxide was useful for cytoplasmic fibrillae and flagella, provided that the smear was kept always moist. Other authors (Schmid and Kamniker, 1926; Westphal, 1935; Wenrich, 1939) have made use of dried smears, and by their methods I have produced preparations resembling some of their illustrations. After comparing these with wet smears, I am convinced that the damage done in drying is a dangerous source of error, all the more misleading because a dried film can often be stained more easily than a wet one. Most of this work is based on smears fixed for 15–30 minutes in modifications of Flemming's or Champy's fluids. The variants most commonly used were: (1) Flemming—1 per cent. chromic acid, 16 parts; 2 per cent. osmium tetroxide, 4 parts; glacial acetic acid, 1 part; and (2) Champy—3 per cent. potassium dichromate, 2 parts; 1 per cent. chromic acid, 2 parts; 2 per cent. osmium tetroxide, 1 part. (Unless otherwise stated, the terms 'Flemming' and 'Champy' in this paper refer to these modifications and not the original formulae.) Flemming gave the clearer general picture, including that of the nucleus, and Champy beautifully preserved the cytoplasm. Feulgen's 'nucleal' reaction has been tried; after fixation in Flemming or osmic vapour and hydrolysis in weak hydrochloric acid for 15–20 minutes or 7 minutes respectively, and staining for 3–4 hours, some positive results were obtained, but as an aid to morphological study the method was useless. I believe that the fault lies in the material rather than the technique, for I have obtained better results with similarly treated *T. muris*. The standard method of hardening fixed material in 96 per cent. alcohol for 48 hours, mordanting all day in iron alum and staining all night in Heidenhain's haematoxylin (both in aqueous solution), has given by far the most useful results. In addition, cytoplasmic inclusions have been studied with the aid of Janus Green and the Champy-Kull and Benda techniques for mitochondria.

It is necessary to say a word in explanation of the style of illustration chosen. *T. vaginalis* is a tiny, delicate, and complex organism, admittedly difficult to fix and stain. It has often been found impossible to judge from the more or less diagrammatic black and white pictures of some authors whether their confident assertions about minute and crowded structures were supported by their preparations. I have thought it best to supply portraits of individual flagellates as faithful as I could make them and leave the reader to assess their value as evidence for both my conclusions and my doubts.

III. General Observations and Feeding Methods

1. *The Appearance of the Living Organism*

T. vaginalis is already sufficiently well known to require no lengthy general description. In length it varies from 6 to 30 μ exclusive of the projecting portion of the axostyle and when measured in its natural position as it lies

freely in liquid. If correctly fed its size is maintained in culture. When undistorted by pressure, it is a plump, spindle-shaped flagellate, with four anterior flagella, a short undulating membrane, and an axostyle which often projects posteriorly (Pl. 1, Figs. 1, 2, and 5). Its cytoplasm contains numerous round, refringent granules which have been the subject of comment ever since Künstler, in 1884, mistook them for ingested bacteria. Almost every author notes that they tend to be arranged in several rows about the axostyle and the basal fibre of the undulating membrane. Now *T. vaginalis* is, as noted by Donné (1836), an exceedingly active and highly polymorphic organism, constantly stretching, contracting, and bending almost double, with strong flexures of the axostyle (Pl. 1, Figs. 2 and 3). These contortions imply that its cytoplasm is usually in a state of violent agitation, in the midst of which the axostyle and other fibres offer situations of relative stability about which the granules may repose. Granules remote from these skeletal structures are irregularly arranged and their agitation as the animal moves can be observed; the regular arrangement about the fibres is probably a simple result of the dynamics of their environment.

Twice in a vaginal discharge and frequently in culture, exceptionally large forms have been seen with plump, immobile bodies and a small, clear, mobile anterior region bearing the flagella and membrane. The largest and least normal of them exhibited long immobile lobes or 'fingers' of hyaline cytoplasm and were degenerating. According to Hogue (1944), who made a special study of these forms, they have the peculiar habit of autotomizing the large posterior region. She considered them to be produced by the physical properties of the semi-solid medium she employed, but I have invariably found numerous smaller, normal trichomonads accompanying the abnormal types. From the fact that they occurred only in exceptionally heavy infections or in culture tubes with abnormally high populations, I am inclined to regard them as hypertrophied individuals produced by overfeeding in a specially nutritious environment.

2. *The Feeding Methods*

Setting aside the erroneous views of Künstler (1884), who mistook the axostyle for an oesophageal tube, we find that later authors give the impression of great uncertainty about the cytostome and feeding (Reuling, 1921; Hegner, 1925; Wenrich, 1931, 1935, 1939). Schmid and Kamniker (1926) thought that a cleft at the anterior end of the body between their two blepharoplasts might have been a cytostome. They put forward the unusual view that the flagella were withdrawn into this cleft and protruded, on stimulation by foreign bodies, to fish for food; leucocytes were supposed to be ingested by pseudopodia. Their observations on living material were supported by some on flagellates fixed by drying and stained as Gram smears which cannot be accepted as evidence for their isolated opinions. Bland *et al.* (1931) included a cytostome in their diagram of *T. vaginalis*, but omitted it without comment from their paper of the following year. All these authors (except Schmid and

Kamniker) agreed that food inclusions were rarely seen in the cytoplasm and Reuling thought that feeding might be osmotic. Bland *et al.* (1932) found little evidence that *T. vaginalis* ingested bacteria in nature, though they did so in culture. Powell (1936) believed that there was no cytostome and supported his views with a clear description of feeding, with most of which, as far as it goes, I am in agreement. *T. hominis* has a cytostome through which it feeds on small bacteria (Dobell and O'Connor, 1921).

    T. vaginalis feeds on small particles such as bacteria and the detritus derived from broken-down coagulated egg and on larger objects such as erythrocytes and starch granules and probably on food in solution. Three main methods can be distinguished. (i) When feeding on bacteria and other minute particles in the liquid part of its environment, *T. vaginalis* usually attaches itself by its axostyle to a cell or other firm base with its body leaning out into the liquid, constantly extending and contracting and twisting, with flagella and undulating membrane incessantly working. There is no regular food stream directed to any constant point on the body. The flagella do not beat one after the other in series but all together, like the thongs of a knout, usually away from the undulating membrane, but sometimes towards it. By their action, bacteria are knocked against the body, usually posteriorly; some are carried there by the membrane, but this does not seem to set up any well-marked food current or to have any special importance in feeding. Most, if not all, of the particles slip off, but some are recovered by the flagella which knock them back again and again on to the body and, sooner or later, one of them adheres to the surface. It is noticeable that when a particular bacterium is receiving attention, the apparently accidental blows of the flagella may be supplemented by stretching and bending movements of the body as if the flagellate were reaching out for the particle. Eventually small groups of bacteria and other particles make tiny mounds attached to the body surface. Very slowly, over periods of an hour and more, they disappear. I have never seen any pseudopodial action during this process, which is so gradual that it is impossible to say, from direct observation, whether disappearance of the particles indicates that ingestion has taken place, but minute inclusions, though rare, are sometimes seen in the bodies of *T. vaginalis* and some particles must at times be ingested, though probably not as a general rule. (ii) Large objects, such as starch grains, are dealt with by a method which is a modification of that already described, but in which the flagellate makes far greater use of its body to hold the object. It wraps itself round as much of the grain as possible, at the same time clasping it with its flagella, which move only slightly unless the grip is lost, when they knock it back into position with repeated blows. From time to time the flagellate may shift by relaxing its hold and sliding round the grain to attack it from a new angle. After prolonging this treatment for over an hour, trichomonads have been seen, on straightening out, to have ingested small starch grains which distorted their cytoplasm. (iii) *T. vaginalis* burrows deeply into the soft debris found in the bottom of culture tubes (Bland *et al.*, 1932).

Individuals have frequently been seen, quite motionless, and partly hidden in semi-solid accumulations of egg debris; if some movement of the surrounding liquid disturbed them, it was noted when they emerged that granules of food material were adhering to them; they had evidently been feeding without the use of their flagella. At no stage in any of these processes have I ever seen a cytostome in use, nor can I identify one with certainty in fixed preparations.

The following facts appear to support the view that extracellular digestion is common among these flagellates. Food inclusions have very rarely been seen, and this is in agreement with the observations of most other workers; yet all flagellates capture bacteria and, indeed, spend almost all their time doing so; no strains ignore them. (Bland *et al.*, 1932, held that adherent bacteria were symptoms of 'lowered vitality', but they offered no supporting evidence and the universality of the phenomenon among even my healthiest stocks argues against their supposition.) Strains which do not ingest starch grains pay attention to them in the way described. This might be attributed to the exercise of a common reaction, however unsuccessful, to food material or to solid particles of the right size; but, as already explained, the addition of starch improves cultures of strains which do not ingest it. Similarly, human erythrocytes were treated like starch grains by strains which did not ingest them and, on being discarded, they were seen to be distorted in outline although untouched corpuscles on the same slide retained their normal appearance. Finally, as Westphal (1935) pointed out, the adherence of particles is not due to the general stickiness of the body surface; it is the symptom of a localized, special, physiological condition such as would be produced by a secretion at the point of adherence.

IV. STRUCTURE AND DIVISION

1. *Cytological Structure*

In stained preparations, the most conspicuous feature of *T. vaginalis* is usually the large number of granules already mentioned (Fig. 3). They vary slightly in size and more in number and distribution. They fill up much space and obscure most of the other cytoplasmic structures as well as the nucleus. Unfortunately, they are best preserved by those fixatives which least distort the rest of the body. After fixation with Champy or Flemming without acetic (original and modified formulae), they are spherical and stain very deeply with iron haematoxylin. Acetic acid does not completely destroy them, but commonly attacks them so that, after Bouin or Schaudinn fixation, they are less clear-cut and stain less deeply, appearing sometimes as obscure dark blots; in such preparations, where preservation of the granules is imperfect, there is a constant danger that they may be mistaken for food inclusions, and when, as very frequently, they surround the nucleus, it is most difficult to distinguish the outline and contents of that organ; conclusions based on methods likely to be affected by this source of error should be considered

with great caution. Champy-Kull preparations, with or without post-chroming, showed the granules brilliantly stained with acid fuchsin. After staining by Benda's alizarin crystal-violet aniline water method, the granules were violet. Apart from the difference in colour, the appearance of the granules after these last two treatments is that shown in Fig. 3. Although these facts were consistent with the view that the granules were mitochondria, it was never possible to stain them vitally with Janus Green (1 in 10,000 for upwards of an hour).

The 'resting' nucleus is oval, with a delicate membrane, the outline of which is not reinforced by any marginal concentration of chromatic material. There is a single karyosome, usually surrounded by a slight halo (Figs. 1, 1 A, and 2). (The term 'karyosome' is here used descriptively as applied to similar nuclear granules in other trichomonads and without implying more about its homology and nature than stated in this paper.) The rest of the nucleus stains only faintly and is probably structureless; a slight unevenness in its consistency is most probably an artifact. Neither karyosome nor membrane stains strongly after Champy; both are more evident after Flemming. I have never found any trace of the karyosome in Feulgen preparations in which dividing nuclei were stained and I believe the 'resting' phase to be Feulgen negative. This stage is relatively uncommon, most of the nuclei being in one of the phases preparatory to division; for this reason, and because there is little to stain in the nucleus, it is easily overlooked.

Immediately before the nucleus, at the anterior tip of the animal, lies the blepharoplast. It is a deeply staining, compound structure, usually lobed so as to suggest, especially in almost completely destained specimens, that it consists of four granules overlapping each other. During the early stages of division, when the constituent granules separate a little, the four can be distinctly counted (Figs. 4 and 7), but whether they remain as discrete entities throughout the trophic phase I have been unable to determine; they were too tightly packed. From the blepharoplast, the flagella, undulating membrane and its basal fibre, the axostyle and the parabasal fibre take their origin. According to Powell (1936), the blepharoplast always consists of five granules to which these motor and skeletal elements, as well as a rhizoplast, are all related in a 'very definite and constant fashion'. After many attempts to follow him, I find it impossible to make any such positive statement about the arrangement of these tiny and crowded objects. I believe that ordinarily the axostyle is attached to a granule on one side of the blepharoplast, and, on the other side, the axoneme of the undulating membrane and its fibre to a second, and the parabasal fibre to a third granule (Fig. 4); all the flagella are not attached to the same granule, but I am uncertain to which they belong. I am unable to find the rhizoplast or fifth blepharoplastic granule. This description of the relations of the blepharoplast to its fibres, so far as it goes, has been checked frequently in trophic organisms and is consistent with the behaviour of the granules and organellae during division; no evidence against it has been observed.

The four flagella are usually at least as long as the body of a fully grown trichomonad and their length is fairly constant, so that they seem disproportionately long in small animals. They often appear to be proximally joined in pairs for a short distance. I can find no support for Andrews's contention that one pair is slightly longer than the other. I have occasionally come across specimens of *T. vaginalis* which seemed to have only three flagella; these were in smears fixed in Champy or Flemming, not in living material or osmic fixed smears—which were very reliable for flagella counts— and I attach little importance to them.

The undulating membrane, as all recent authors agree, is short and confined to the anterior half of the body, and its axoneme is not continued posteriorly as a free flagellum. This I have found to be true of all fresh material and, with two exceptions, of cultivated trichomonads. The exceptions were both primary cultures, in Ere, with, in one case, the addition of 0·5 per cent. sodium citrate to the liquid component of the medium; the populations were low and the flagellates rather small. The first sign of abnormality was noted on the fourth and fifth days respectively; in most of the flagellates the membrane was exceptionally long. On the following day it had grown right round the body as far as the axostyle and a few flagellates had a long, trailing flagellum leaving the body at the end of the membrane and hanging limply beside the posterior, protruded length of the axostyle. Next day nearly all the flagellates were in this condition and the trailing flagellum was about as long as the body; it was maintained in sub-culture. I had not at that time discovered the use of dextrose and was unable to produce flourishing cultures from which preparations could be made. The history of these two strains seems to be similar to that of Andrews', except that in mine the flagellum was a little slower in developing. Forty-six other strains kept for periods of 8 days to 5 months and examined for this point failed to show any significant variation in the length of the membrane. Nor was anything resembling a *Eutrichomastix* phase ever seen; probably the shortness of the membrane in *T. vaginalis* explains the absence of those accidentally produced forms—common enough in trichomonads like *T. muris* with long, powerful membranes—in which the axoneme is torn away from the body right up to the blepharoplast, thus imitating the structure of *Eutrichomastix*.

A slender, deeply staining basal fibre—shorter and much more delicate than the long, thick rod which is so prominent a feature of *T. hominis* preparations—leaves the blepharoplast and runs below the undulating membrane (Figs. 1, 2, 4, and 5). It is a skeletal structure; in both living and fixed material individuals have been seen in which the fibre was pulled out from its normal position and, hinging on the blepharoplast and carrying the membrane with it, stretched the cytoplasm between it and the axostyle as the skeleton of a bird's wing stretches the patagial membrane.

The flexible axostyle leaves the blepharoplast to curve round the nucleus on the opposite side to the undulating membrane, and then, if at rest, usually

straightens out to run directly to the posterior end of the organism from which it may protrude (Fig. 1). Variations in its position may be seen in Figs. 2, 3, and 5, where, if the whole extent of the axostyle is not visible, its course is indicated by the granules around it. In small flagellates reared without additional carbohydrate, the exposed posterior portion was relatively long. The axostyle is a slender, siderophile structure, only very slightly broader anteriorly than posteriorly and of about the same width and appearance, except for length, as the basal fibre; it was always very easily distinguishable from the thick, tapering rod, with siderophile edges and hyaline core, found in such forms as *T. hominis* and *T. muris*.

A third siderophile fibre, of variable length, arises from the blepharoplast and curves round one side of the nucleus, between it and the basal fibre, towards the axostyle. Below the nucleus it may cross the axostyle and continue for some way beyond it before tapering to an end (Figs. 1, 1 A, 2, and 5); sometimes it fails to reach as far as the axostyle and occasionally it ends at its point of contact with that organ, so as to give the false impression —apparently that which misled Reuling (1921)—that it forms one side of an axostyle anteriorly expanded to embrace the nucleus. Like the basal fibre and the axostyle, it is flexible and is variously curved by the movements of the body. This delicate, deeply staining structure, which often resembles the basal fibre so closely as to suggest a duplication of it, is the parabasal fibre. There is no difficulty about its demonstration and no dispute as to its existence; it has never been described in *T. hominis*. The same certainty does not apply to the parabasal body of *T. vaginalis*, the presence of which has been affirmed, denied, or ignored by different authors (see p. 94). After fixation in Flemming or Champy, prolonged staining in iron haematoxylin (24 hours each in mordant and dye), and appropriate differentiation, the parabasal body is stained grey, and this is the most reliable method of demonstrating it. In Benda preparations it is often visible, stained purple, and it may also be stained by leaving smears overnight in Delafield's haematoxylin, diluted with distilled water to about one-twentieth of its normal strength. After fixation in Schaudinn, the body is very rarely demonstrable with any degree of certainty. The staining reactions of the parabasal body are admittedly somewhat variable and, in smears differentiated to show nuclear detail, it is often completely decolorized in every specimen; except in dried smears, which I distrust, I have never seen it so sharply defined as it appears in some of the illustrations published by Wenrich and his collaborators (Wenrich, 1931, 1939; Bland *et al.*, 1931). But, in spite of its apparent absence in some smears, and some uncertainty about its identification in others, it is recognizable in too many individuals, especially after the first treatment described above, for it to be dismissed as an artifact. In appearance the parabasal body is a band of lightly staining material of rather uneven texture, adhering to the parabasal fibre, along which it stretches for a variable length, rarely less than half-way from the blepharoplast and usually not quite to the tip. It is ordinarily continuous (Figs. 1 and 2), but sometimes

broken up into separate patches of stainable material (Fig. 1 A). Its most common appearance is that of some thick, coagulated, semifluid substance accumulated about the parabasal fibre and present in varying quantities in different individuals. I have occasionally found specimens with two parabasal bodies, each related to its own fibre. Wenrich has suggested that such individuals are preparing for division, but to this I cannot assent. Normal dividing forms possess only a single parabasal fibre up to a late stage in the process, when a second fibre arises *de novo*. The forms figured by Wenrich and those seen by me are more likely cases of abnormal duplication; doubling of various structures is a fairly common abnormality of this and other trichomonads (see below, and Bishop, 1931).

2. Division

Very little about the division of *T. vaginalis* is to be found in the literature. It will be understood that, although dividing stages are common enough in heavy infections and good cultures, few of these are of any use to the investigator. The great majority have their nuclei and other structures obscured by the granules already mentioned. Also, it is usually impossible so to differentiate stained preparations that the conditions of the nucleus and cytoplasmic organs are accurately revealed in the same specimen, e.g. the flagellates shown in Figs. 7–9 accurately display the nucleus, but are unreliable for fibrillae, while that in Fig. 17 shows the reverse state.

The first indication that the flagellate will divide is the appearance within the nucleus of chromatic granules outside the karyosome. They increase in number and the nucleus elongates. For a while the faintly staining karyosome is still discernible (Figs. 4 and 5), but it soon disappears. The nucleus now reacts positively to the Feulgen technique (Fig. 6), and continues to do so throughout division. It is very difficult to count the granules accurately, but there are about twenty. At about the same time the tight complex of blepharoplastic granules is slightly relaxed (Figs. 4 and 7), but, typically, it is the nucleus which shows the first signs of division. These changes result in the appearance most commonly found in stained smears and frequently figured in the literature, though not previously recognized for what it is; I conclude that the phase is prolonged. The body of the dividing trichomonad now usually rounds off, but occasionally an elongated, more or less oval form is retained up to the time of cytoplasmic fission. The following stages are rarely seen and presumably take place rapidly. The nuclear membrane disappears, but the chromatic contents of the nucleus lie in a clear, pale area distinct from the surrounding cytoplasm. The numerous small granules begin to associate in groups, at first rather indefinitely (Fig. 7), but in the end five small aggregates are found, in which the granules are at first plainly separate but finally begin to lose their identity (Figs. 8 and 9); in this way five more or less ellipsoidal chromosomes, all about the same size, are formed (Fig. 11). My opinion is based on the small number of individuals in which the nuclear structure during these stages was perfectly clear; the

chromosomes, when formed, usually overlapped, but in a few cases no confusion was possible and then five chromosomes could be distinctly counted (Fig. 11); views of earlier and later stages in division were consistent with the opinion that five is the correct number of chromosomes. Meanwhile, a dynamic and rapid reorganization of the cytoplasmic structures takes place in a way difficult to follow, as the fibrillae lose their distinctive characters; always very similar in appearance, they stain only weakly in the earlier stages of division, and, at the same time, they shorten, except for the parabasal fibre, which seems to retain its normal length and is thus usually the longest of the fibrillae at this stage. Division of the blepharoplast takes place by the dissociation of its constituent granules during prophase; one granule divides and its two daughters, the centrosomes, pass to opposite poles of the nucleus; they remain attached by a stout, deeply staining centrodesmus. Of the remaining granules, two remain associated with one centrosome and one goes with the other (Figs. 8 and 9). The cytoplasmic fibrillae accompany the granules to which they are attached, so that the shortened axostyle passes to one pole, while the undulating membrane and its basal fibre, with the parabasal fibre, go to the other (Fig. 10). The separation of these organellae follows the course expected from their arrangement about the 'resting nucleus'. There is no good reason to suppose that any of the fibrillae disappear at any time during division; their presence, though not their identity, is often faintly revealed in more deeply stained individuals than those portrayed in Figs. 8, 9, 11, and 12, and, when they again become recognizable, they are found to occupy the positions in which they were last plainly visible (Fig. 14). The flagella are divided between the daughter blepharoplasts at the same time as the fibrillae, but I am unable to explain precisely how they are disposed.

I have not seen any arrangement of the chromosomes which constituted a typical metaphase; there is no spindle or equatorial plate, and, perhaps correlated with their absence, the chromosomes do not divide simultaneously, but irregularly; they apparently split transversely (Figs. 12 and 13), their daughters passing rapidly to opposite poles, while the centrodesmus elongates (Fig. 15). The chromosomes very soon lose their individuality and the commonest telophase picture shows that they have disintegrated into granules like those seen in early prophase (Fig. 16). This is the last stage to give a positive Feulgen reaction. By this time each blepharoplast has three granules in addition to the centrosome (Fig. 16). The new fibrillae appear suddenly; they grow out *de novo* from the blepharoplast, and there is nothing to suggest that any of the old fibrillae divides. In smears in which the parabasal body was consistently stained, dividing forms showed that a single body was present at one pole towards the end of prophase (Fig. 10), while some telophase figures had one at each pole (Fig. 17); I have found no evidence that the parabasal body is self-perpetuating.

Division of the cytoplasm is longitudinal and is initiated by the appearance of a cleft between the two sets of organellae, so that the body of the parent is heart-shaped, with a set of organellae in each lobe of the heart. During this

time the dividing organism is very active, with flagella and membranes working vigorously, feeds, and, like trophic forms, is highly polymorphic. At no stage could any sustained pull between the two daughters be observed; both seemed to behave as independently as their attachment allowed. Their movements gradually deepened the cleft until they were connected by only a slender thread. This finally snapped, apparently as the accidental result of some particularly violent movement of one or both daughters, and division was complete.

I am unable to offer any account of the fate of the centrodesmus. It is present up to the last moment before final separation of the daughter organisms. The appearance of forms such as those shown in Figs. 15 and 16 suggests that when the centrodesmus snaps each of its halves completes the number of fibrillae required by the new flagellate, and for a long time I sought confirmation of this view. Instead I have found forms like that in Fig. 17, which have a full complement of fibrillae, though some not yet fully grown, at each pole, as well as a centrodesmus. Presumably, therefore, the latter is resorbed, as in *Trichomonas caviae* Dav. (Grassé and Faure, 1939), though it seems probable that the centrosomes are incorporated into the blepharoplasts as fourth granules (Fig. 16).

Various abnormalities, presumably the results of irregular fission, occur in *T. vaginalis* as in other members of its genus. Of these the commonest are (*a*) flagellates with two sets of organellae, including the nucleus, (*b*) those with two or even three additional nuclei, usually in the middle or posterior part of the body, but with only a single set of cytoplasmic organellae. Dislocation of the synchrony between nuclear and cytoplasmic division leads to occasional forms in which the nucleus reaches the end of prophase without any sign of division of the blepharoplast. Abnormal nuclei, containing a confused mass of chromatic blocks, clumped closely together, or with numerous large round granules in mulberry formation, also occur: I can offer no explanation of them. I have never observed in *T. vaginalis* the curious multiple forms illustrated by Bishop (1931, Fig. 60).

V. DISCUSSION

In separating the species of human trichomonads, previous authors have made use of differences in size and such characters as the structure of the nucleus, axostyle, undulating membrane, parabasal apparatus, and other cytoplasmic inclusions. In regard to most of these points there is surprisingly little agreement.

Size. *T. vaginalis* is the largest trichomonad found in man, with a length, excluding the axostyle, sometimes reaching 30 μ. The greatest length recorded for an intestinal trichomonad seems to be 20 μ for *T. hominis* (Dobell and O'Connor, 1921) and for '*Pentatrichomonas ardin-delteili*' (Wenrich, 1931; Powell, 1936). *T. tenax* is smaller, with maxima of 12μ (Bland *et al.*, 1931) or 17 μ (Powell, l.c.). The large size of *T. vaginalis* may be maintained in culture and the fact that starved specimens may be reduced to the dimen-

sions of *T. hominis* (Andrews, 1929) is not significant. I can find no evidence that there may be two size races of *T. vaginalis* (Bland *et al.*, 1931); even the authors themselves admitted that the size distinctions disappeared in culture.

Nucleus. The most serious defect in the existing accounts of *T. vaginalis* is lack of consistency in descriptions of the nucleus. It has a karyosome (Bensen, 1910; Reuling, 1921); it sometimes has one (Hegner, 1925; Wenrich, 1931); none could be found (Powell, 1936); Bland *et al.* (1932) figured four distinct types of nuclear structure for this species. Some misunderstandings have undoubtedly arisen from failure to recognize early stages in mitosis for what they were; others, e.g. references to a structureless nucleus, seem due to faulty technique or observation. In specimens with unobscured and clearly stained 'resting' nuclei, I find that a karyosome is always recognizable. *T. hominis* has a similar nucleus, though its karyosome and nuclear membrane are more conspicuous. The fine extra-karyosomatic granules seen rarely by Bishop (1931) but thought by Dobell and O'Connor (1921) to be an ordinary feature of the nucleus of *T. hominis* may well mark the earliest preparations for division in that species, as they do in *T. vaginalis*. However that may be, the 'resting' nuclei of both species are almost identical. The nucleus of *T. tenax*, containing several large chromatic masses, is easily distinguishable from that of *T. vaginalis* (Hinshaw, 1926).

Axostyle. According to Reuling (1921) the axostyle consists of two fibrillae which embrace the nucleus (probably the axostyle and parabasal fibre) and two more from the nucleus itself. Hegner (1925) saw the axostyle as a single, slender fibre, but thought he might have been examining the edge of what was really a thick, hyaline rod. He was possibly influenced by the fact that the latter is the commonest type of axostyle in trichomonads; it occurs in *T. hominis* (Dobell and O'Connor, 1921; Bishop, 1931) and has been attributed to *T. vaginalis* by Wenyon (1926), Andrews (1929), Powell (1936), and by Wenrich and his collaborators. In a later paper Wenrich (1939) asserted that the axostyle was composed of two to eight fibrillae, which, in abnormal individuals, became more or less separated and distinct; the phenomenon was said to be especially clear in dried films. I believe that the axostyle, as revealed in wet preparations, is a single, deeply staining fibre, similar to the basal fibre (Figs. 2 and 5). Its structure was correctly given by Westphal (1935), who realized that in this respect *T. vaginalis* differed from *T. hominis*. Hinshaw (1926) described the axostyle of *T. tenax* as a slender siderophile rod; it resembles that of *T. vaginalis*. It is worth noting that when the new axostyles of *T. hominis* first appear during division they are slender fibres (Bishop, 1931, Fig. 54) with an unmistakable resemblance to the new axostyles of *T. vaginalis* (Fig. 17); the stout, hyaline structure seems to be secondary.

Undulating membrane. All workers, except Künstler (1884), are agreed that the undulating membrane and its supporting basal fibre are normally short, extending about half-way or slightly less down the body from the blepharoplast. Since Andrews (1929) reported that in culture her specimens

developed a long membrane and, from it, a free posterior flagellum, other
investigators have occasionally seen the long membrane, but only Westphal
(1935) found the posterior flagellum, and that but rarely. Two whole strains
of mine grew a long membrane and posterior free flagellum, but I have never
found such forms, as Westphal reported them, as exceptional individuals in
otherwise normal populations. Bland *et al.* (1932) and Powell (1936) thought
that the long membranes in such specimens of *T. vaginalis* belonged to trun-
cated individuals which had autotomized their posterior ends. Against this
explanation as a general rule may be argued (1) that autotomy is associated
with degeneration (Hinshaw, 1926; Hogue, 1944) and healthy forms may
have full-length membranes, and (2) that in my two strains, as in Andrews',
the development of the long membrane was gradual and occurred in animals
no smaller than those with normal membranes. It must be borne in mind
that this phenomenon has never been seen in nature and, in my experience,
is rare in culture. The occasional development of this similarity to *T.
hominis* may indicate phylogenetic affinity, but is not a proof of specific
identity any more than the resemblance between the membranes of *T.
vaginalis* and *T. tenax* proves *their* identity. According to Westphal (1935)
and Wenrich (1939), between the margin of the membrane and the axoneme
a more delicate filament is to be found in dried preparations, but only rarely
in wet ones. Similar filaments are said to be demonstrable by Bodian's
protein-silver technique in '*Pentatrichomonas hominis*' (Kirby, 1945) and a
trichomonad presumed to be *T. limacis* Dujardin (Kozloff, 1945). Basing
my opinion on wet preparations, I am unable to depart from the conservative
view that the axoneme forms the margin of the membrane in *T. vaginalis*.

Little is known about the *parabasal apparatus* of human trichomonads.
There seems to be no doubt that, though Powell (1936) was unable to find
the chromophobe body, it does occur in *T. vaginalis*, as Wenrich and his
co-workers have consistently maintained. But their use of this structure in
separating *T. vaginalis* from *T. hominis* is difficult to support. We know
practically nothing about it in the latter species; Dobell (1942), in a paper
on staining technique, incidentally comments that he has stained the para-
basal body of *T. hominis*; Kirby (1945) has suggested that a small, ellipsoidal
body lying near the nucleus, and which he impregnated with silver, might
be a parabasal body. Even apart from these dubious circumstances it seems
injudicious to employ in taxonomy a character demonstrable only with diffi-
culty. The parabasal fibre is a different matter; it is easily seen in all ordinary
preparations of *T. vaginalis* and has never been described in *T. hominis*.

The large cytoplasmic granules of *T. vaginalis* closely resemble mito-
chondria in their reactions to fixatives and stains. The addition of small
quantities of acetic acid to fixatives did not destroy them, though it usually
impaired their affinity for stains; it is already known that some mitochondria
are resistant to weak concentrations of acetic acid (Nicholson, 1916). No
technique has so far revealed any other inclusions which could possibly be
interpreted as mitochondria. On the other hand, the granules failed to stain

with Janus Green. Hogue (1922), relying exclusively on Janus Green, described the mitochondria of *T. hominis* as a few short, blunt rods. Whatever decision may eventually be reached about the granules in *T. vaginalis*, their treatment by Wenrich (1931, 1939) as specific characters seems open to the same objections as his similar treatment of the parabasal body.

Division. Apart from a few isolated sketches, the only previous account of the division of *T. vaginalis* is the admittedly incomplete one by Powell (1936). The earliest stages in *T. vaginalis* agree in general with those described by Bishop (1931) in trichomonads from the gut of man and *Macacus nemestrinus*. In all, as the karyosome disappears, chromatic material becomes recognizable outside it, but the large number of small granules in *T. vaginalis* contrasts sharply with the small number of larger granules in Bishop's material. As early prophase is prolonged this stage is commonly met with and the appearance of the nucleus is then characteristic of *T. vaginalis*. According to Powell, this species has four chromosomes. I am alive to the dangers of contradiction on such a point in this difficult material, but feel some confidence in correcting the figure to five. Wherever all the chromosomes could be clearly and separately observed there were five of them, and there were always five aggregates of granules in such stages as those in Figs. 8 and 9. Less convincing views of the nucleus in these stages, though not decisive, often supported my interpretation. Moreover, I have tried Powell's method of fixation with very hot Bouin and find it difficult to understand the deformations produced by it.

Complete demonstration of the relations of the blepharoplast has eluded me. It consists of four granules and one supplies the centrosomes by division. Partition of the parent's fibrillae to their poles and growth of the new basal fibre and undulating membrane take place somewhat earlier in *T. hominis* than in *T. vaginalis* and *T. tenax*. In all species the new axostyle is formed relatively late. There is no division of the old axostyle, but in *T. hominis* the old one is absorbed and two new ones grow out from the blepharoplasts. Hinshaw (1926) thought that this was true of *T. tenax* also, but he was admittedly swayed by evidence from other species, and his illustrations show the old axostyle still present at anaphase and perhaps even at telophase. I have never found convincing evidence that the old axostyle totally disappears during the division of *T. vaginalis* and believe that it persists, though shortened and with a greatly reduced affinity for stains. I have not discovered how the full complement of granules is restored to each daughter blepharoplast; some, at least, of the new flagella are, as in *T. tenax*, the last organs to appear, and it seems likely that they are related to the new granules.

The systematic status of the vaginal trichomonads of man. The work of Dobell (1934) mentioned on p. 80 provides very convincing evidence of the identity of the strains of *Trichomonas* he employed, but it is of great importance to note that these did not include any from the human vagina; the author himself suggested that the taxonomic question would only be settled by experiments on human beings. It is my contention that it has

been settled by the disclosure of constant structural differences between *T. hominis* and *T. vaginalis* of sufficient significance to justify their separation as good species. When fully grown, *T. vaginalis* is larger than any other human trichomonad ever is. Its nuclear organization differs unmistakably from that of *T. tenax* and, if its life-history be pursued into prophase, just as clearly from that of *T. hominis*; numerous prophase nuclei are found in almost every smear and it should not be difficult to avoid confusion. The thick, long, heavily staining basal rod which is such a striking feature of *T. hominis* is replaced in *T. vaginalis* by a shorter and much more delicate, though well-defined, fibre. The fibrillar axostyle of *T. vaginalis* resembles that of *T. tenax* and is quite different from the stout, hyaline rod with siderophile edges found in *T. hominis*. The foregoing points are so constant and so easily demonstrable that they may be properly used in diagnosis. In addition, *T. vaginalis* differs from *T. hominis* in some ways which, either because they are imperfectly understood in one species or another or because they are difficult to observe, may be used only to augment a case for differentiation established independently; here might be included the structure of the parabasal apparatus, length of the undulating membrane, presence or absence of a free posterior flagellum, and nature of the cytoplasmic inclusions. It might, of course, still be maintained (*a*) that *T. vaginalis* and *T. hominis* may each survive in the typical habitat of the other, and (*b*) that if so they would then lose their specific characters. With regard to the former possibility, whatever view is taken of the evidence against it, no serious evidence in favour of it exists; the latter possibility is mere conjecture. In these circumstances, and taking into consideration the structural differences between these trichomonads, the onus of proof lies on any who still maintain that they are specifically identical.

VI. SUMMARY

1. *Trichomonas vaginalis* has been cultivated on various media and a simple method of maintaining cultures on Boeck and Drbohlav's egg-Ringer-albumen medium is described.

2. The feeding methods of the flagellates in culture have been studied and some evidence of extracellular digestion is recorded.

3. When cultivated on media deficient in carbohydrate, *T. vaginalis* is reduced to the dimensions of *T. hominis*, but when adequately fed it main-

tains its distinctively larger size. Out of forty-eight strains of *T. vaginalis*, only two developed in culture the long undulating membrane and free posterior flagellum typical of *T. hominis* in nature.

4. The structure of *T. vaginalis* has been reinvestigated. (i) It differs constantly and significantly from the intestinal trichomonads of man in size, nuclear organization, and the form of the axostyle and basal fibre. (ii) It also differs from *T. hominis* in the characters of its parabasal apparatus and cytoplasmic inclusions, and, in nature, in the length of its undulating membrane and in lacking a free posterior flagellum, but for reasons discussed in the text these points are at present considered less reliable in diagnosis than those given under 4 (i).

5. It is concluded that *T. vaginalis* is a species distinct from all other human trichomonads.

6. The method of division has been described. In general, it follows the same course as *T. hominis*, but separation of the old, and growth of the new, cytoplasmic structures occurs somewhat later in *T. vaginalis*, and the old axostyle is retained throughout division. There are five chromosomes, formed during prophase from aggregations of extra-karyosomatic granules.

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EXPLANATION OF PLATE I

All figures are portraits of *Trichomonas vaginalis* made with the aid of a Leitz cam. lucida, and are ×2,350. Except where otherwise stated they were stained in Heidenhain's iron haematoxylin. Figs. 7–9 and 11–13 were differentiated to show nuclear detail and are unreliable guides to cytoplasmic structure; the reverse applies to Figs. 10 and 17.

Abbreviations: *Ch.*, Champy's fluid. *Feul.*, Feulgen's reaction. *Fl.*, Flemming's fluid. *Os.*, Osmic vapour.

Fig. 1. Trophic form with continuous parabasal body. *Fl.*

Fig. 1 A. Trophic form, anterior end only, with discontinuous parabasal body. *Fl.*

Fig. 2. Trophic form; note coagulated appearance of parabasal body. *Fl.*

Fig. 3. Trophic form, showing cytoplasmic granules. *Ch.*

Fig. 4. Anterior end of an individual preparing for division; extra-karyosomatic granules are appearing in the nucleus and the blepharoplastic components are dissociating. *Fl.*

Fig. 5. Slightly later stage than the last, with more numerous granules, and the karyosome disappearing. *Fl.*

Fig. 6. Prophase form showing only the nucleus, now without a karyosome. *Os. Feul.*

Fig. 7. Beginning of chromosome formation, with granules associating in indefinite groups. Note the blepharoplast. *Fl.*

Fig. 8. Five groups of granules recognizable and the centrosomes are disjoined. *Fl.*

Fig. 9. A slightly later stage than the last. Note centrosomes and blepharoplastic granules. Part of body omitted. *Fl.*

Fig. 10. Approximately the same stage as the last, more deeply stained. At one pole are the membrane and its shortened basal fibre, the parabasal body, and its now relatively long fibre; at the other pole is the shortened axostyle. Part of the body omitted. *Fl.*

Fig. 11. End of prophase. Five chromosomes plainly visible. *Fl.*

Fig. 12. Beginning of anaphase; two chromosomes are dividing transversely. Part of body omitted. *Fl.*

Fig. 13. Slightly later stage than the last. All the chromosomes are splitting. Note traces of the fibrillae. *Fl.*

Fig. 14. Approximately the same stage as Fig. 12, more deeply stained. At one pole are the short axostyle, one flagellum, and the root of another; at the other pole are the short, faintly indicated basal fibre and its membrane, in deeper focus than the plainer, longer, parabasal fibre. Cf. Fig. 10. *Fl.*

Fig. 15. Beginning of telophase. Traces of the chromosomes still visible. At one pole are the new membrane and basal fibre above and the old axostyle below the nucleus; at the other pole are the new axostyle above and the old parabasal fibre, still comparatively long, and the old membrane and basal fibre below the nucleus. *Fl.*

Fig. 16. Slightly later stage than the last, with nucleus resuming the prophase condition. Cytoplasmic structures probably as in the last, but insufficiently stained. Note blepharoplastic granules. *Fl.*

Fig. 17. Late telophase (?), deeply stained. At one pole, the old parabasal fibre, twisted partly out of focus, basal fibre and membrane below the nucleus, and above it the short, new axostyle; at the other pole, the old axostyle, regaining its length and staining affinities, new basal fibre and membrane and the short, new parabasal fibre. At both poles, the parabasal body is just evident. *Fl*

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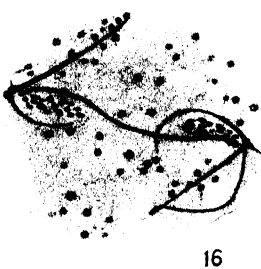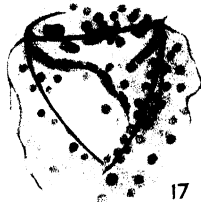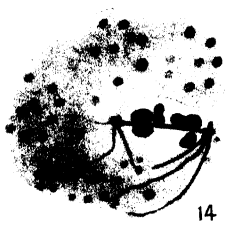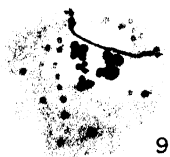
PLATE I

Amoeba kerrii (n. sp.): Morphology, Cytology, and Life-History

BY

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With one Plate and five Text-figures

INTRODUCTION

IN June 1939 a collection of plants from the fresh-water pools on the shore, in the vicinity of the Marine Biological Station, Keppel, was brought to me by Miss Maureen McAlister. Since there were no crustaceans in the water it was stored as a possible source of protozoa (Taylor, 1920). The decaying material produced no crustaceans, a good omen. A few boiled wheat grains were added as a pabulum to supply the needs of the infusors which by this time were numerous, and Glasgow tap-water was used to compensate for evaporation of the original liquid. A glass plate covered the culture. In the summer of 1941 a more detailed examination of the material revealed the presence of amoebae easily visible under the low power of a Greenough binocular. The extreme opacity of these amoebae was arresting, the cause of it due to the fact that each was densely packed with cytoplasmic inclusions including large numbers of crystals. The nucleus when in a position to be seen stood out quite sharply, looking almost like a vacuole by contrast with the blackish cytoplasm. Further search revealed individuals not quite so black in reflected light, probably because younger.

METHODS OF CULTIVATION

The original culture having been well stirred, several c.c. of it were put into a Petri-dish (4 in. diameter) with 4 or 5 wheat grains and water. After 3 months these cultures yielded no amoebae. One or two more trials also resulted in failure to establish Petri-dish cultures by this method. I next tried a technique which succeeds well in the case of *A. proteus* Y, i.e. of taking a large bulk of the original culture with 10 to 20 wheat grains and water (bulk about 2 litres) and placing these in a cylindrical glass trough (8 in. in diameter by 4 in.). One only of these trials succeeded. However, the contents of the numerous failures were stored and did eventually produce a few amoebae. The reason for the failure of the above technique will be obvious when the life-history is described, and the best method of securing spectacular cultures will be given below under the account of fission divisions.

IDENTIFICATION AND CLASSIFICATION

In the light of much experience gained by a long study of large fresh-water amoebae I concluded that the dusky individual was new to science and that it belonged to Schaeffer's genus *Metachaos* (Schaeffer, 1926). I propose, however, to retain the name *Amoeba*, see below, for the genus and to give the specific name *kerrii*, in honour of my one-time teacher, now Sir John Graham Kerr, who long ago persistently urged the importance, for teaching purposes, of an investigation of the life-history and cultivation of the amoeba commonly called *A. proteus*.

My reasons for retaining the name *Amoeba* are:

1. Our scant knowledge of the life-history of free-living amoebae. More knowledge may modify a classification based on the study of the adult. For example, while *A. lescherae* and *A. proteus Y* would be placed in the genus *Chaos* because both possess longitudinal folds in the ectoplasm, their developmental forms are very different one from the other (see Taylor and Hayes, 1944, Text-fig. 5). On the other hand, the developmental forms of *A. discoides* and *A. kerrii* confirm the relationship based on a study of the adults.

2. The fact that the name *Amoeba* has become firmly embedded in the English language, and that Schaeffer's nomenclature has not yet been generally accepted even in U.S.A.

As will be shown later, the life-cycle of *A. kerrii* occupies 8 or 9 months. Apart from the developmental stages it presents quite remarkably differing appearances, these differences being due to age and physiological conditions. Full-grown individuals are those usually chosen by authors for descriptive purposes, but these vary. Therefore some standard for comparative purposes is essential. Taylor and Hayes (1944) suggest that the young adult should be chosen, this stage being defined as that in which fission division is regularly occurring. In no amoeba I have yet studied is the contrast between the mature and senile adult so great as in this. And since the longevity of the adult *A. kerrii* is considerable this aged adult may well preponderate in any 'catch' made during the summer and autumn in the open.

MORPHOLOGY OF MATURE TO SENILE ADULT STAGES

When transferred to a slide the amoeba of this stage reluctantly attaches itself and begins to creep by a few stout rounded, broad pseudopodia. The endoplasm is packed with crystals (Pl. 1); cuboid, square prisms, truncated bi-pyramidal in shape, which mask the presence of the large nutritive spheres, food vacuoles, and other cytoplasmic inclusions. Longitudinal folds of the ectoplasm are not present, hence the inclusion of *A. kerrii* in the 'Group' *Metachaos* (Schaeffer, 1926). The tip of a pseudopodium often bifurcates; the pseudopodia sometimes produce globular masses along the two sides. The ectoplasm is not voluminous, the contents of the endoplasm coming up close to the periphery. A cross-section of the middle region of a pseudopodium would be almost semicircular in outline.

The nucleus (Pl. 1) is always easily distinguishable in a creeping individual, being sometimes lenticular in shape but quickly turning over into a disk shape and then back again to the lenticular, as the creature progresses. The nuclear sap is very mobile. One gets the impression that the nucleus is large in comparison with the cytoplasm but it is difficult to measure the ratio (see below). Measurements of adult amoebae stained and mounted are 525 by 150μ, 375 by 105μ, 450 by 300μ; they are somewhat flattened out by the cover-slip.

In order to obtain some ratios between size of nucleus and cytoplasm, several amoebae of the same age were transferred to a slide and gently warmed. This had the effect of making each specimen more or less spherical. In this condition they were fixed, stained, and mounted. The ratio of the diameter of the nucleus to that of the spherical amoeba was so varied as to negative any comparative calculation of ratio. Moreover, as in *A. lescherae*, so in *A. kerrii*, the diameter of a spherical uninucleate differs little from that of a binucleate or even a 4-nucleated individual. Voegtlin and Chalkley (1930–45) obtained measurements by gently stimulating the amoeba by drawing and withdrawing it from a capillary tube until it had assumed a spherical form. They measured three dimensions of the nucleus and calculated for an ellipsoid. They concluded that volume of amoeba increases as the ratio of the cytoplasm to nucleus increases. But no corresponding measurements are available to give a comparative account of the cytoplasm nucleus ratio in all the large fresh-water amoebae.

When viewed *in situ* in the Petri-dish culture the majority of these stages, i.e. mature to senile, are floating.

THE YOUNG TO MATURE ADULT STAGES (Pl. 1)

These individuals have a greater surface area than obtains in the senile. They spread more rapidly and move more quickly when put on a slide under a cover-slip. The shape varies considerably. There is frequent reversal of direction in the flowing endoplasm of the various pseudopodia, an advancing pseudopodium often yielding its contents to another. The width of the advancing pseudopodium is often greater than that of the others. The young adult is much less dusky than the senile, crystals and nutritive spheres being smaller. Therefore at this stage *A. kerrii* bears a greater likeness to *A. proteus* Y, *A. lescherae*, *A. discoides*. There is often a little tuft of rounded, short, pseudopodia-like processes at the hind end which is reminiscent of the villi which give its name to *A. villosa*. In the latter amoeba, however, this structure is permanent. In *A. kerrii* this uroid is used as a pivot and appears in very young individuals a short time after they have hatched. The ectoplasm is more voluminous than in old individuals; a slight web can sometimes be recognized between adjacent pseudopodia. Two distinct regions can be recognized in the endoplasm of a rapidly moving amoeba under a cover-slip: a centrally placed, more densely packed, more active one, which gives the impression of a deep ravine down the middle of the pseudopodium, and a flanking region where the contents are more sparse and the movement slower.

This moving stream of endoplasm will flow on either side of an obstructing nucleus if the amoeba is well spread out (Hayes, 1938). The width of this active endoplasm varies in different individuals.

When given more freedom (e.g. studied in a live-box) with the help of a binocular eyepiece the pseudopodia of *A. kerrii* are seen to be arranged in definite tiers more or less parallel to the long axis. The contents of an advancing pseudopodium may be reversed or it may flow into a pseudopodium in a higher plane while a new pseudopodium is forming in a plane nearest the substratum. A criss-cross system of flowing endoplasm is the apparent result. But although the progressing and reversing streams of endoplasm are being continually developed, the amoeba as a whole disappears from the field of view. Sometimes the endoplasm ceases to flow and becomes piled up in one area giving the amoeba a lumpy appearance.

When studied *in situ* in Petri-dish culture with the help of a binocular eyepiece it can be observed that comparatively little of the amoeba is fastened to the substratum, a fact revealed by the passage of micro-organisms under the amoeba. The uroid, and peripheral portions of some pseudopodia, anchor the creature, while other pseudopodia emerge in all planes. Here again reversal of flow as well as progression are observable, and as before the amoeba goes out of the field of view. Feeding and moving phases alternate. Most of the food vacuoles are formed on the under side but are discernible from the upper side. The amoeba remains stationary while feeding sometimes for an hour or so. When the captured food organisms are dead the amoeba begins to move about actively again.

A Contractile Vacuole (Pl. 1) lies behind the nucleus. It evacuates very deliberately, and often, before this event, the newly forming vacuole is well on its way to attaining its maximum size. In one stained specimen the respective diameters of the old and newly forming vacuoles were 36μ and 28μ. Other measurements of the diameter of the contractile vacuole are 42μ, 44μ, 29μ, 39μ, the size depending on the age of the amoeba.

A. kerrii, by contrast with the other large amoebae, is not a voracious feeder under normal conditions. I have observed one specimen which lived in a damp chamber for $3\frac{1}{2}$ weeks without food. The food organisms are ciliates, flagellates, rotifers. As in other related amoebae it possesses nutritive spheres which are small in young amoebae, larger in older ones, and can attain a diameter of $6\cdot5\mu$.

The cytoplasm has a closer texture than that of *A. proteus Y* or *A. lescherae* and stains more deeply. It is much less vacuolated than the latter and much less easily ruptured than the former. This is especially well seen when a preparation containing several well-spread amoebae is irrigated with methyl green or aceto-carmine. When the fixative penetrates slowly great bladder-like distensions of the ectoplasm and a corresponding compression of the endoplasm occur. In some cases 'lines of force', very similar to those seen in the cytoplasm when a nucleus is dividing, are to be seen in this distended ectoplasm which rarely ruptures.

villi

contractile
vacuole

nutritive
sphere

nucleus

food
vacuole

crystal
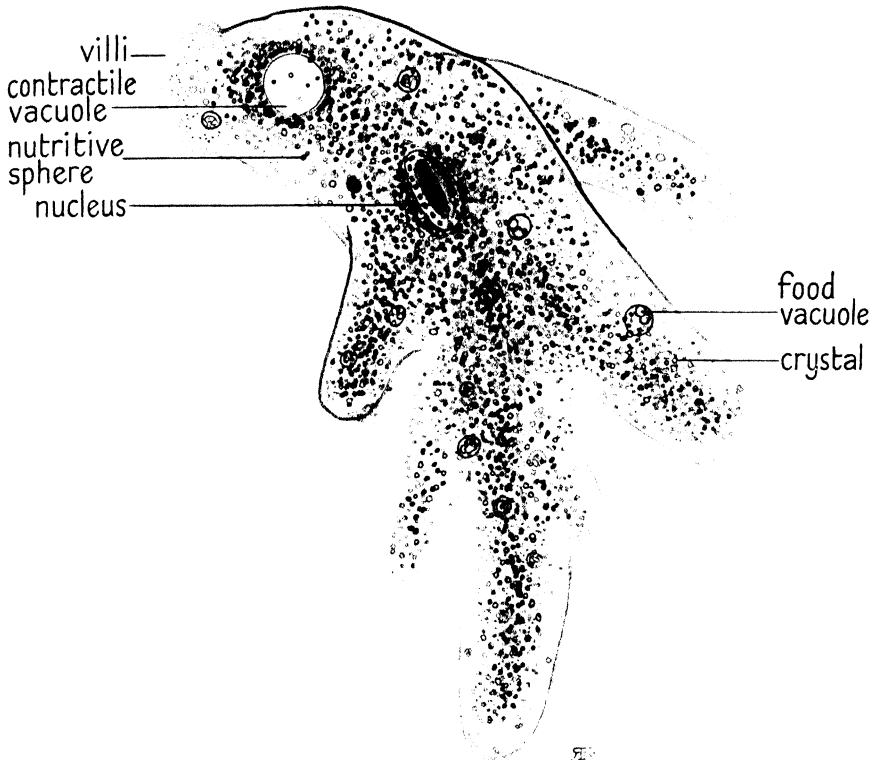
Freehand drawing of Amoeba kerrii (young adult)

The nucleus changes its form very readily, as previously stated. It has the same general plan of structure as that of the other species of amoebae (Taylor, 1930; Hayes, 1938) and will be described more fully later. It can be dissected away from the cytoplasm when its membrane has reached its maximum thickness.

When a 2-month-old Petri-dish culture is scrutinized under the lower powers of a Greenough binocular most of the individuals are of a more or less radiate type, some floating, some creeping. But there are also to be seen fission-spheres (see later) and a few outsized spherical individuals. These are multinucleate and are most numerous in an ageing culture. These outsized spherical multinucleate forms seem to be characteristic of all classes of amoebae—Clifford Dobell records them in parasitic amoebae. Their significance is obscure (see Taylor and Hayes, 1944, and Levy, 1924 and 1928).

ACETIC ALCOHOL AND ACETO-CARMINE STUDIES

Aceto-carmine alone, and aceto-carmine after fixation in acetic alcohol, are valuable and quick reagents for the study of amoebae. At the outset these were employed. It was found that the cytoplasm of *A. kerrii* stains very deeply, a fact which militates against the usefulness of aceto-carmine as a nuclear stain. Fortunately the crystals are dissolved out by these reagents. Thus the remaining cytoplasmic contents can be more easily studied. In the light of one's former experience of amoeba nuclei, the most unlooked for result of aceto-carmine studies was the varying degrees of prominence exhibited by the nuclei of *A. kerrii*. Generally such an outstanding object in other amoebae, the nucleus of *A. kerrii* could sometimes be distinguished with difficulty from the surrounding cytoplasm. This was not always the case, however, especially when the nuclear membrane was stout. Lest these varying appearances were due to chemicals dissolved out of the numerous crystals, Bouin was employed as a fixative and the specimens were well washed in alcohol, which readily dissolves the crystals, and stained in Ehrlich's haematoxylin. The results confirmed the aceto-carmine investigation, some nuclei being outstanding and possessing thick nuclear membranes, in yet others the nuclear membrane was so thin as to be hardly discernible and the contents of the nucleus not sharply differentiated from the cytoplasm.

Aceto-carmine after acetic-alcohol fixation is useful for revealing the nutritive spheres. These lie in a vacuole, have a deep red rim, and a pale slate-coloured interior. The size of the nutritive spheres depends upon the age of the amoeba. An excellent method of demonstrating the abundance and size of the nutritive spheres is to fix and stain the amoeba with methyl green in acetic acid. The nutritive spheres stain green (see Taylor, 1939).

NUCLEUS OF A. KERRII (Text-fig. 1)

The nucleus in the main resembles that of the other large fresh-water amoebae. Its typical form is best seen in the young adult (A). Under the nuclear membrane are regularly arranged blocks containing chromatin

separated by a clear space from a centrally placed disk-shaped karyosome, nuclear sap filling the interstices. The whole is extremely mobile, alternately oval or circular (cf. A and H) in outline as the amoeba creeps, with all manner of intervening shapes as the nucleus changes over from oval to circular 'view'. The karyosome (H) is band-shaped in the former (H) and disk-shaped in the latter (A). A scrutiny of large numbers of amoeba nuclei fixed in Bouin and stained in Ehrlich's haematoxylin reveals many interesting
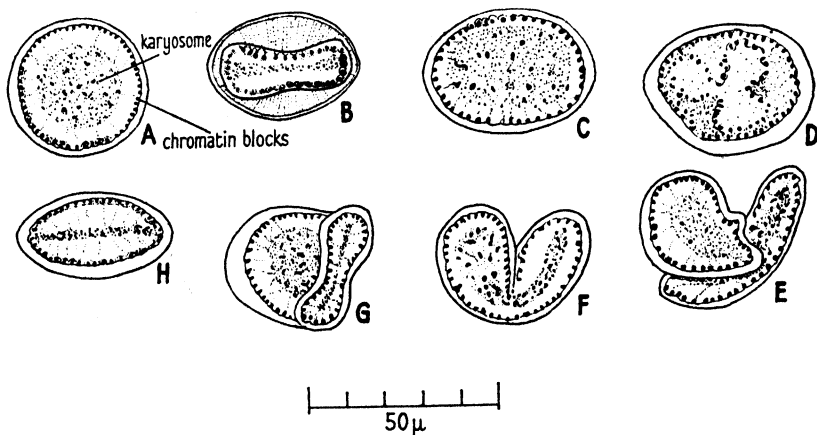
TEXT-FIG. I. Nucleus of *Amoeba kerrii*

A. Typical nucleus of a very young adult. Surface view. B. Nucleus changing into 'side-view' position. C. Karyosome close up to chromatin blocks. D. Karyosome much vacuolated. E. Nucleus from an old individual, suggestive of amitotic division. F. A common appearance of the nucleus of an old adult. G. Nucleus rolling into 'surface-view' position. H. Nucleus in 'side-view' position.

diversities other than that of outline. The most striking, as already mentioned, is the varied reaction to stain, and the varying thickness of the nuclear membrane. As already mentioned, when the latter is thick the nucleus can be removed entire from the cytoplasm. In ageing amoebae the nucleus tends to become deformed (D, E, F), the outline corrugated. 8-shaped figures are common and all appearances which so strongly suggest amitotic division are to be found.

FISSION DIVISIONS IN A. KERRII (Text-fig. 2)

In July 1943 a number of square, solid watch-glasses was assembled and into each was placed an adult amoeba taken at random from the parent culture along with a quantity of the culture fluid and food organisms. In every case the amoeba failed to divide. It however lived on for a varying period of time. These negative results were puzzling, especially as I had detected binucleate specimens in the stained preparations. Next I prepared cultures of the flagellates and ciliates found in the parent stock and upon which the amoebae were feeding and procured very young adults for the

inoculation. Each amoeba was carefully washed and introduced into the watch-glass with a few food organisms and some fresh water. The next day most of the watch-glasses possessed two amoebae. Fission division obviously occurs only in the young adults. Once initiated, repeated fission produced a little colony of amoebae in each watch-glass. When the number approached about sixty-four I removed them to a Petri-dish (4 in. in diameter by ½ in.) supplying fresh water and food organisms and so obtained spectacular cultures. It was then found possible to add boiled wheat grains as a pabulum and so obviate the necessity of cultivating the food organisms separately. By subculturing I increased the number of my cultures, since
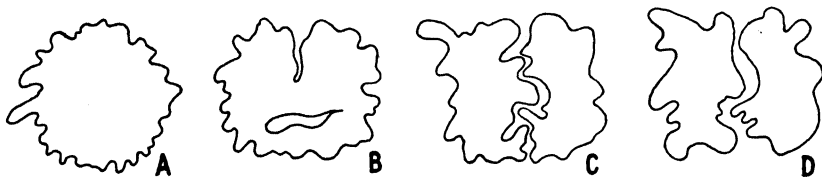
TEXT-FIG. 2. Fission of Cytoplasm

A. Fission-sphere.   B. Division has extended to equator of sphere.   C. Division complete.
D. Two daughter amoebae separate.

access to almost unlimited numbers is necessary for the complete elucidation of the life-history.

Fission of the Cytoplasm (Text-fig. 2) can easily be witnessed *in situ* or on a slide by removing one of the rounded morula-like individuals (= fission-spheres) from the culture (A). These fission-spheres adhere to the substratum while dividing in contrast to what obtains in *A. lescherae*. The break between the two daughter amoebae never stretches uninterruptedly from pole to pole (B) like a meridian, but lesser breaks along the meridian can be detected as the pseudopodia of each daughter amoeba form, and extricate themselves from each other (C). In one observation the whole process lasted half an hour. The two daughter amoebae remain very near each other for a time, again in contrast to *A. lescherae* where the two daughter components are very soon indistinguishable from the other members of the culture. At no time is there a long strand of protoplasm connecting the two as is so often figured in text-books.

MITOSIS

The first record of mitosis in the large free-living amoebae was made by Sister Bernardine Carter (Carter, 1913). Since then it has been demonstrated in *A. proteus* (Dawson, &c., 1937), *A. discoides* (Taylor and Hayes, 1942), *A. lescherae* (Taylor and Hayes, 1944), *A. dubia* (Dawson, &c., 1935).

Method of obtaining Material to demonstrate Mitosis

Secure at least a dozen rich Petri-dish cultures where the amoebae are multiplying rapidly. Keep them as cold as possible until two or three hours

before they are required, then raise the temperature to 70° or 80° F. Dividing amoebae will be present in sufficiently large numbers to warrant the making of permanent preparations. Remove each fission-sphere with a fine pipette to a slide. After providing each slide with three or four individuals in a minimum amount of liquid, gently lower a cover-slip provided with beeswax feet. The amoebae will grip the substratum. Irrigate quickly with Bouin's fluid, replace this by 90 per cent. alcohol, giving a good soaking in this and changing the alcohol in order to dissolve the crystals. After 70 per cent. alcohol stain in Ehrlich's haematoxylin, differentiate in acid alcohol, dehydrate in cellosolve, clear in xylene, and mount in Canada balsam. Examples of mitosis, if in a favourable position in the fission-sphere, stand out quite clearly under a No. 7 objective. Many of the mounted spheres show no nucleus; in yet others the mitotic nucleus is only discovered after much searching. In the former case the nucleus is deeply buried or masked by an overlying food vacuole. In any case the early prophase stages are difficult to see. The nuclear membrane is thin, and since the chromatin in an amoeba nucleus is very sparse, the early prophase is very often almost invisible (Text-fig. 3 A). The nucleus, of course, may be so placed on the slide as to be seen from the side, or foreshortened, but in a full-face view the outline is like that of a barrel. All the achromatic material gradually becomes arranged on a series of meridional lines, each composed of thick and thin lengths of material. Later (Text-fig. 3 B) these become little corkscrew-shaped masses as the chromosomes condense and make their way to the equator. Out of these corkscrew masses the definitive spindle-fibres are differentiated. These develop from the equator (B) and gradually spread to the poles. There is, of course, a great variety in the size of the dividing nuclei as there is in the resting ones, the size depending on the age of the amoeba and its volume, the larger the amoeba the larger the nucleus.

When the chromosomes are fully condensed at metaphase they lie on the equator in a clear area filled with a fluid-like substance that stains a bright pink in Ehrlich's haematoxylin. This surrounding pink area can be detected right up to late telophase. The chromosomes are very small and numerous. In anaphase (C and D) the barrel-shaped nucleus becomes more elongated. A few of the spindle-fibres, like the ribs of a barrel, stand out very conspicuously (D). The chromosomes still lying in the 'pink' area already referred to never reach the roof of the dome-shaped poles of the spindle (D and E) in telophase. In surface view, therefore, the chromosomes lie on the periphery of a circle just inside the nuclear membrane (F) in each daughter nucleus. At first the spindle-fibres can be distinguished (F, *b*), but these are gradually converted into corkscrew masses of achromatic material (F, *a*, G, *a*). The daughter nuclei are well on their way to reconstruction when the fission of the cytoplasm is completed (G, *a* and *b*). The nucleus absorbs liquid and so becomes larger. Around the periphery (G, *a*) the chromosomes can still be distinguished from the achromatic material. They become less distinguishable as the karyosome reforms. The nuclear membrane is thin and the whole
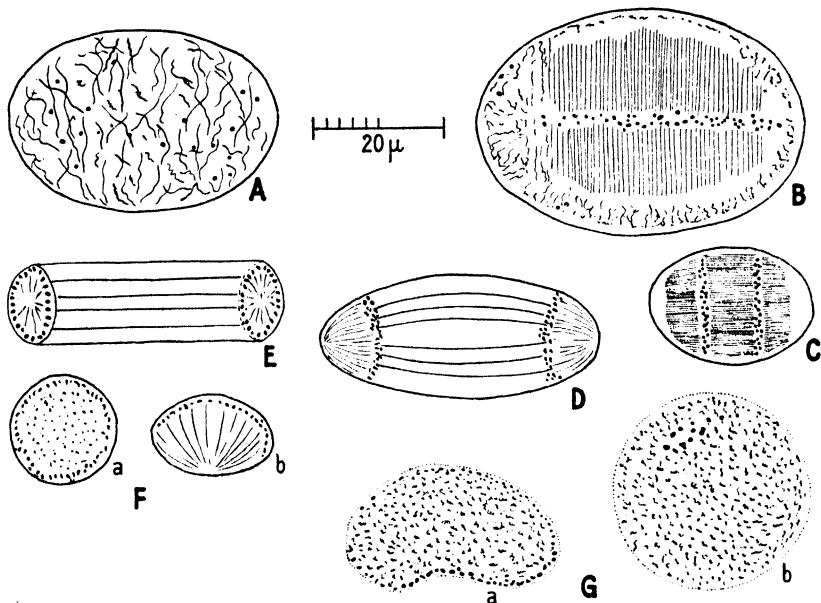
TEXT-FIG. 3. Mitosis in *Amoeba kerrii*

N.B.—The figures have been drawn from fission specimens of varying size.

A. A very early prophase. Achromatic structures lose their staining capacity, become arranged meridianwise: the sparse amount of chromatin makes the nucleus difficult to detect. B. From a large amoeba at later prophase. Chromosomes condensing out at equator, spindle-fibres becoming differentiated from the equator towards periphery.— Distinction between spindle-fibres and undifferentiated achromatic elements very pronounced. C. Early anaphase. Chromosomes lie in a clear area which stains a bright pink in Ehrlich's haematoxylin. D. Early telophase.—Some spindle fibres stand out very clearly.—The polar caps seen in side-view—spindle-fibres clearly marked. The area around the chromosomes stains bright pink. E. Telophase.—Polar caps seen in end view. Spindle-fibres more palely stained. Chromosomes around periphery still conspicuous. F. Daughter nuclei in an undivided amoeba. In *a*, end view of polar cap, the spindle-fibres have reverted back to achromatic fibres. In *b* they can still be detected. The coloured, clear region around the chromosomes makes them still a conspicuous object. G (*a* and *b*). From an amoeba fixed as soon as the fission of the cytoplasm was completed and examined in aceto-carmine. Nuclear membrane so thin as to be almost invisible. Chromosomes, in their pink-coloured area becoming less distinctly differentiated—the rest of the nucleus a clearly stained, homogeneous mass of discrete elements.

difficultly distinguishable from the surrounding cytoplasm. (N.B. This want of prominence of the nucleus must not be confused with that described previously.)

It is interesting to note that in a large number of the rounded off, large, multinucleate amoebae the nuclei are often near the periphery and very close to each other. They have all the appearances of newly constructed nuclei. What causes the failure of the cytoplasm to divide after the nucleus has divided remains still to be explained.

An observation made on a fission division may throw light on the pheno-menon that occurs in all the amoebae I have studied, namely the decrease in its size as the amoeba becomes senile. In old cultures pieces of non-nucleated amoeba-cytoplasm occur. Yet I have never observed any large-scale occurrence of the casting off of lumps of cytoplasm by amoebae which might account for the presence of these non-nucleated fragments. However, on one occasion I had transferred a large fission-sphere of *A. kerrii* to a slide to study fission. The amoeba gave every sign of dividing into two larger and equal protoplasmic masses, and one smaller. The two larger and equal-sized portions were true daughter amoebae—the smaller portion was a non-nucleated mass. This may have been due to the mechanical disturbance involved, though it is not likely, as scores of fission-spheres so transferred have proceeded to divide normally; but it might also explain how ageing amoebae can become progressively smaller.

REPRODUCTIVE CYCLE

In his brilliant and comprehensive work on parasitic amoebae, Dobell (1928, 1938) has found no trace of a sexual cycle. No such cycle has been found in *A. proteus* (= dubia) (Carter, 1915), *A. proteus* Y (Taylor, 1924), *A. discoides* (Hayes, 1938), *A. lescherae* (Taylor and Hayes, 1944), and in the amoeba now being described, i.e. *A. kerrii*. On the advice of Dr. Helen Pixell Goodrich, to whom I have been indebted in the past for much constructive criticism, the clumsy and ugly-sounding nomenclature used in the description of *A. lescherae* (Taylor and Hayes, 1944) and in *A. discoides* (Hayes, 1938) has been abandoned, because of this absence of a sexual cycle. The mature amoeba which produces ripe cysts is referred to simply as a mature amoeba and the newly hatched individuals as amoebulae. (See also *A. discoides* (Quart. J. micr. Sci. **87**, 195), where the nomenclature has been emended.)

The *Fission Cycle* in the life-history of *A. kerrii* is succeeded by that of the *Reproductive Cycle* when each mature amoeba becomes converted into a number of encysted young. The process starts with the emission of chromidia (i.e. small masses of chromatin material on an achromatic base) from the nucleus into the cytoplasm (Text-fig. 4 A) where they become the rudiments of the encysted young. The rudiment grows, as can be seen by studying progressively more mature amoebae. In the meantime the nutritive spheres in those amoebae which are preparing to form encysted young become less numerous, their substance becoming absorbed into the general cytoplasm. Under the influence of the nucleus rudiment cytoplasm is formed around it and eventually a cyst-wall encloses this small nucleated mass, i.e. the encysted young (Text-fig. 4 B and C). Hundreds of these cysts are formed from every ripe adult. Eventually the cytoplasm of the latter disintegrates and the cysts are liberated into the surrounding water where they remain for a period of time.

Method of Procuring Microscopical Preparations in the Reproductive Cycle

Select old well-conditioned individuals from a successful four months' culture, fix in acetic alcohol and then stain in aceto-carmine. Or, fix in aceto-carmine and give several washings of the fixative to dissolve out the crystals. Chromidia and all stages in the formation of encysting amoebae can thus be secured.
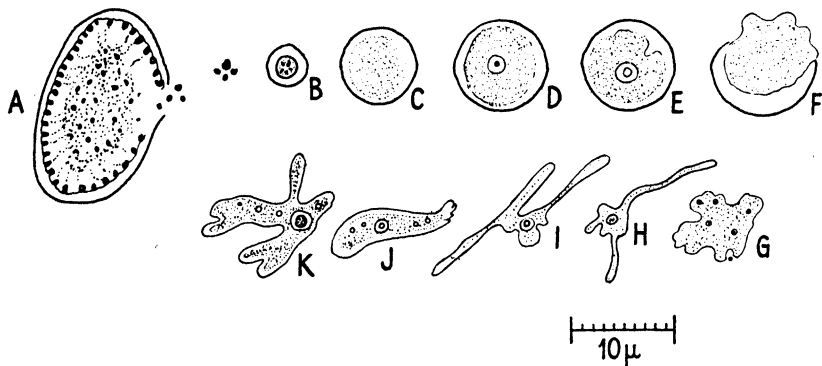
TEXT-FIG. 4. Cyst Formation.—Hatching of young *A. kerrii.*—Early stages of Development A. An adult nucleus.—Nuclear membrane absorbed where chromidia are escaping into cytoplasm of a mature amoeba. B. and C. Differentiation of encysted young amoeba. D. Cyst nearing the time of hatching, nucleus visible. E. Encysted amoeba with a functioning contractile vacuole. F. Escaping amoebula, nucleus not easily visible. G. Newly hatched amoebula feeding—several food vacuoles. H. Ectoplasm has become more fluid. Long anchor-like pseudopodia enable the creature to grip the surrounding debris. I. Older individual. J. Limax-like creeping amoebula. K. Floating amoebula.

EXCYSTATION OF A. KERRII

Method of Procuring Cysts for Excystation

A pure line culture of *A. kerrii* was set up at the end of July 1943 by placing one young adult in a solid watch-glass with culture fluid and water containing food organisms grown separately. When the progeny numbered about sixty-four a Petri-dish was prepared with culture fluid and food organisms for its reception. Food organisms were supplied regularly as required. At the end of August two freshly boiled wheat grains were introduced. The culture was spectacular at the end of October when fresh wheat was added, the old grains being removed to make a subculture, as they were carrying a number of adhering amoebae.

In January 1944 the culture was undergoing a period of depression. Cysts were abundant. These were studied *in situ* by means of a No. 7 water-immersion lens or on a slide. It is a matter of experience to decide which cysts are ready to excyst. I have had specimens under observation for two weeks before hatching took place. A fully differentiated cyst measures 9 μ, though there is a slight variation in size. The wall is stout and in contrast

to the other large amoebae that have been described, it is single (Text-fig. 4 C, D). When the time of hatching approaches the nucleus is easily visible, centrally placed and surrounded by endoplasm which gradually becomes more and more granular. The periphery of the encysted amoeba does not look like an inner cyst-wall; hence, as explained above, the cyst-wall of *A. kerrii* is single.

Shortly before the actual excysting takes place, a contractile vacuole begins to function (Text-fig. 4 E). The amoebula must therefore be imbibing water and the cyst-wall must be ruptured. After some time the contents of the cyst become obscured by a bluish-tinged oily-looking substance, the ectoplasm. Gradually this latter may be seen oozing out of the cyst, which slowly becomes empty as a more or less limax-shaped amoeba escapes. This moves for a distance of about 45μ and commences feeding on very small bacteria present in the water (G). It is extremely well camouflaged as it lies thus for a varying period of time. The empty cyst-wall remains circular in outline for a long time after the escape of the amoebula, a proof of its toughness. However, when the excystation takes place in the direction of the cover-slip the irregular circular outline of the breach can be detected. It measures about 3μ.

In a day or two the excysted amoebulae develop a more fluid ectoplasm (H) and long slender pseudopodia are extruded. These hook on to debris, which fact suggests that they are a device for transporting the amoebulae to new pastures (H and I). However, when placed on a slide these floating forms may be made to creep (J). When viewed *in situ* the floating forms with elongate pseudopodia are seen to change over to the creeping type (J) or round up. The protrusion of blunt pseudopodia first from one side and then from the other as the amoebula progresses, is somewhat explosive and rapid in character.

FURTHER DEVELOPMENT OF A. KERRII (Text-fig. 5 A)

The amoebula grows slowly, the endoplasm becoming provided with inclusions of various kinds. The limax shape of a creeping form of 100μ is superficially similar to that of *A. proteus* Y save that there is no 'sole', i.e. no precursor of longitudinal folds. In it nucleus and contractile vacuole and a small uroid are easily distinguishable. These developing young are much stronger than those of *A. proteus* Y. As in the adult, so in the developing stages, floating and creeping phases alternate.

NUCLEUS OF DEVELOPING A. KERRII (Text-fig. 5)

A karyosome surrounded by a clear area of nuclear sap enclosed in a nuclear membrane is the structure of the nucleus as seen in the living amoeba. Further details are demonstrated by staining methods. The ratio of the diameter of the karyosome to that of the nucleus is greater than in that of *A. proteus* Y, *A. discoides*, and *A. lescherae*. In preparations made with aceto-carmine or permanent preparations stained in Ehrlich's haematoxylin the consistency of the karyosome varies from a distinctly granular to a smooth more or less homogeneous one.

Lying in the clear space between karyosome and nuclear membrane, at a varying distance from the latter, is a collar of achromatic material which eventually becomes apposed to the nuclear membrane. On this collar (Text-fig. 5 B, C, D) are sometimes to be seen distinct granules of chromatin. When this 'collar' has reached the nuclear membrane the developing young amoeba may be considered to have attained the adult stage. The possession
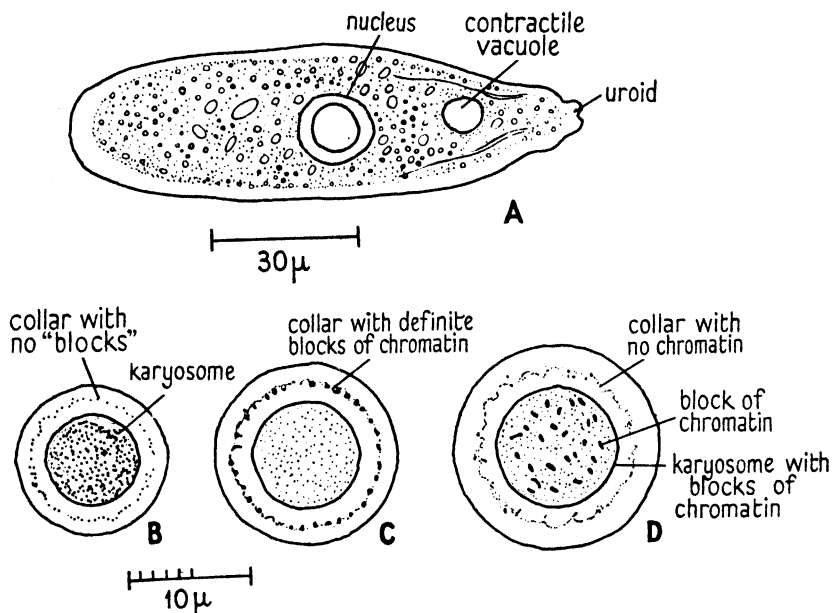
TEXT-FIG. 5.

A. A young amoeba in creeping position. B, C, D. Nuclei of developing *A. kerrii.*

of the 'collar' marks off the immature amoebae of *A. proteus Y, A. discoides, A. lescherae, A. kerrii* from the Mayorellas.

To Mr. Ronald W. Graham Kerr who responded so graciously to my request that he should undertake the filial task of executing the text-figures and plate, I offer my warm thanks and appreciation of his skill.

DIAGNOSIS

Found in ponds. Size increases with age up to a maximum length in locomotion of 576μ. It then decreases as the amoeba becomes senile. The pseudopodia are blunt; few, in senile specimens; the ectoplasm has no longitudinal folds, hence resembles that of *A. discoides* and Schaeffer's genus *Metachaos.* The endoplasm is densely packed with crystals and nutritive spheres giving a more dusky appearance than obtains in the other fresh-water

amoebae yet described. Contractile vacuole about 40μ in a young adult. Food: flagellates, rotifers, ciliates. Floating and creeping forms occur, the former often radiate or spherical except in senile individuals when they are amoeboid. When feeding the amoeba creeps. The species resembles *A. proteus* Y, *A. discoides*, *A lescherae* in the possession of a discoid nucleus presenting a round or band-shaped outline as it is rolled about by the endoplasm. Nuclear division mostly resembles that of *A. discoides*, and in the main, the prophase, metaphase, and anaphase of *A. proteus* Y, but the dome-shaped telophasic elements are in contrast to the cone-shaped ones of *A. lescherae*. Fission divisions are restricted to the young adult and do not occur in ageing individuals.

The species differs from *A. proteus* Y, *A. dubia*, *A. discoides*, *A. lescherae* in its longevity [senile adults linger on in the ponds until winter], in the marked difference between the appearance of a young adult and a senile individual; in the dusky character which is progressively intensified, as old age approaches, by the growth in size of the nutritive spheres and crystals; and by the diminished size of the senile. Another difference is the greater ease with which the reproductive cycle can be traced in *A. kerrii*.

SUMMARY

1. An amoeba characteristically blackish when viewed under the low power of a Greenough binocular in reflected light was discovered and isolated from macerating water weeds found in fresh-water pools on the shore near the Marine Biological Station of Keppel.

2. A study of its morphology revealed its relationship to Schaeffer's genus *Metachaos*. For reasons given above, the name *Amoeba* has been retained for the genus and the specific name *kerrii* has been given in honour of Sir John Graham Kerr.

3. The life-history has been worked out by means of pure-line cultures.

4. The cyst measures about 9μ in diameter. The newly hatched amoebulae grow slowly and develop eventually into young adults which, because of the small size of their crystals and nutritive spheres, are much less dusky than the mature individuals first discovered. An average adult size in creeping is 504 by 576μ.

5. The nucleus, which divides by mitosis, has the same general build as that of *A. discoides*, *A. proteus* Y, *A. lescherae*. Immediately under the nuclear membrane is an achromatic network containing chromatin blocks and connected with a more or less centrally placed karyosome, the whole immersed in nuclear sap. The nucleus assumes varying shapes as it is rolled about by the streaming endoplasm.

6. Morula-like spheres (= the fission-spheres) give rise to two daughter amoebae by cleavage along a meridional central plane. Fission is confined to young adults.

7. A fission cycle is succeeded by a reproductive cycle. Any mature amoeba can give rise to encysted young which are formed by the action of

chromidia escaping from the nucleus. The cyst-wall is single. Hundreds of encysted amoebulae may be formed from one mature amoeba. No sexual stages occur.

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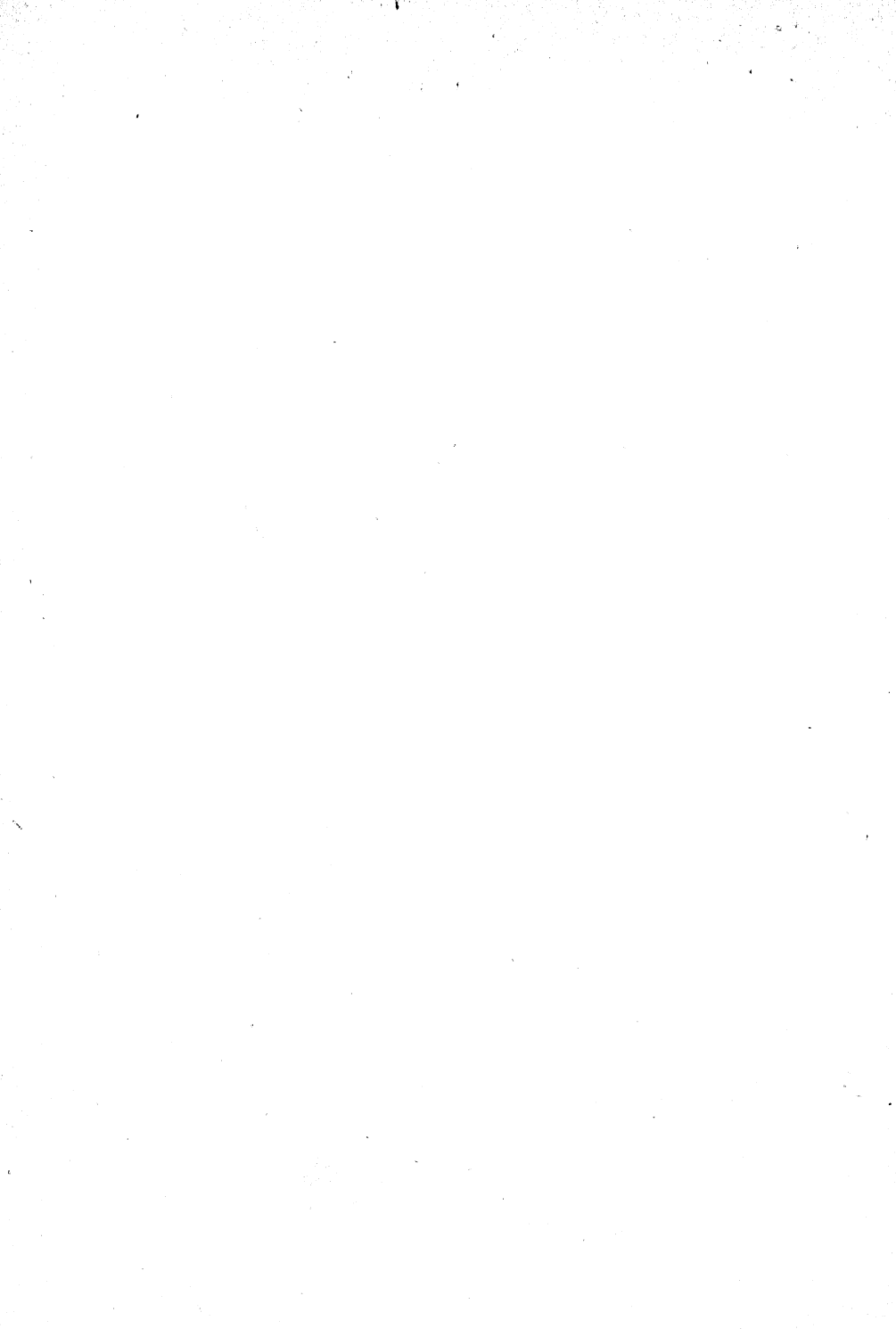
The Histochemical Recognition of Certain Guanidine Derivatives

BY

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CONTENTS

INTRODUCTION

SAKAGUCHI'S test for arginine depends on the production of a pink or red colour when certain guanidine derivatives are treated with α-naphthol and sodium hypochlorite in alkaline solution (Sakaguchi, 1925). This test had been familiar to biochemists for 19 years before it was independently adapted to histochemical use in the same year by Serra (1944) and myself (1944). Our results were published on opposite sides of the battle-front. The purpose of the present paper is to discuss the validity of Sakaguchi's test and to present my histochemical adaptation of it in its latest form.

THE VALIDITY OF SAKAGUCHI'S TEST

Sakaguchi's test was applied to a number of substances by himself and by Poller (1926). The chemical composition of the red substances formed in the test is uncertain. Sakaguchi gives an analysis of the reaction-product with glycocyamine, but Poller questions the correctness of his formula.

Sakaguchi thought that his test gave a positive reaction with all substances having this structure:

$$H\text{---}N=C\diagdown\begin{matrix}\diagup NH_2 \\ N\text{---fatty acid} \\ | \\ H\end{matrix}$$
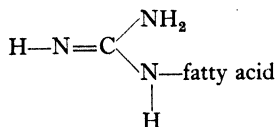
but not with any substance in which the hydrogen atoms shown in the formula are replaced by any other atoms or groups of atoms.

Poller showed that the hydrogen of the H—N= group, and *one only* of the

hydrogens of the NH₂ group might be substituted by methyl, without interfering with the positive reaction. He also showed that a number of substances containing no fatty acid radicle give a positive reaction.

Weber (1930), who did not test any substance not previously tested by Sakaguchi or Poller, drew the tentative conclusion that a positive reaction is given by all substances having the formula:

$$H—N=C\begin{cases} NH_2 \\ N—X \\ | \\ H \end{cases}$$
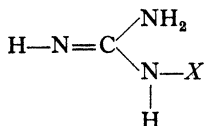
where X is either a fatty acid or an alkyl radicle. This, however, does not cover all known cases. For instance, the hydrogen of the $H—N=$ group may be substituted by methyl, as in trimethylguanidine, yet a positive reaction is given. Further, a positive reaction is given by dicyandiamide, in which X is neither a fatty acid nor an alkyl group.

A study of all the accurately named substances investigated by Sakaguchi and Poller shows that a generalization can be made as to the chemical formula of positively reacting substances, more exact than those made by either of these authors or by Weber. The following substances react positively:

Dicyandiamide, monomethyl-, dimethyl-, and trimethyl-guanidine, glycocyamine, alacreatine, α-guanidino-*n*-butyric acid, galegine, agmatine, and arginine.

Negative results are given by:

Guanidine, asymmetrical dimethyl- and trimethyl-guanidine, creatine, creatinine, alacreatinine, glycocyamidine, nitroguanidine, nitroarginine, hydantoin, methyl hydantoin, hydantoic acid, thiohydantoic acid, biuret, urea, uric acid, allantoin, alloxan, guanine, cyanuric acid, histidine, tryptophane, asparagine, tyrosine, lysine, and ornithine. (Poller also obtained negative results with a number of unnamed nitrogenous compounds, including various other mono- and diamino-acids, but the absence of particulars prevents this information from being used in forming a generalization.)

The facts given above are covered by the following generalization:

A positive result with Sakaguchi's test is given by all substances, so far as is known, having the following formula:

$$\alpha—N=C\begin{cases} H \\ | \\ N—\beta \\ \gamma \\ N—C—\delta \\ | \quad | \\ H \quad \epsilon \end{cases}$$
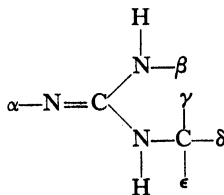
where α is H or CH_3 and β is also H or CH_3. A positive result is not given, so far as is known, by any substance in which this is not so.

The three valences represented by the lines connecting a carbon atom with γ, δ, and ϵ may be satisfied by various atoms or groups of atoms, or all three may be taken up by a single nitrogen atom, in dicyandiamide.

In the present state of knowledge, a positive reaction with anything occurring in any plant or animal tissue must be regarded as indicating the presence of arginine (free or combined), galegine, or agmatine. If further research shows that an appreciable amount of any of the other positively-reacting substances is present in any particular tissue, this conclusion must be modified accordingly. Arginine is, of course, one of the most widely distributed substances in the tissues of organisms, since it occurs in most proteins. Galegine and agmatine, on the contrary, are extremely restricted in distribution. The former was discovered by Tanret (1914) in the seeds of *Galega officinalis* (Leguminosae) and has not been found in other plants. Agmatine was discovered by Kossel (1910) in the testis of the herring. It was subsequently found by Engeland and Kutscher (1910) in ergot. It has also been reported by Kiesel (1924) in ears of rye that are free from ergot. In the present state of knowledge it is reasonable to regard a positive reaction with the test as indicating the presence of arginine (free or combined), except in studies of *Galega officinalis*, ergot, ears of Gramineae, and the testes of the herring and allied fishes.

The Adaptation of Sakaguchi's Test to Histochemical Use

General Remarks

The test succeeds best with paraffin sections. The alkali, however, tends to loosen the sections from the slide, and it is therefore best to attach them with a film of celloidin.

In Sakaguchi's original test the α-naphthol was used at a very low concentration, because it tends to become yellowish in the absence of arginine, and this discoloration is evident when the solution is viewed in considerable thickness in a test-tube. In the test described below, only a thin section is examined, and free α-naphthol is not present in appreciable amount when the observation of colour is made. There is therefore no advantage in using an extremely dilute solution.

The amount of sodium hypochlorite used was determined from the following considerations. If much is used, colour develops very rapidly, but the excess of hypochlorite soon destroys the colour. The object was therefore to find the minimum necessary to produce the full colour. Since ordinary hypochlorite solutions are very unstable, the proprietary product 'Milton' was used. This contains sodium hypochlorite at approximately 1 per cent., together with sodium chloride and small quantities of other salts. Milton is remarkably stable, and the salts other than sodium hypochlorite do not affect the test. Where Milton is not available, a strong solution of sodium hypochlorite should be diluted until it gives full coloration in approximately fifteen minutes, when substituted for Milton in the test described below.

For the quantitative determination of arginine it is desirable to have very

rapid development of colour, and for this reason Weber (1930) substituted sodium hypobromite for hypochlorite. The rapid coloration given by hypobromite is followed by rapid fading, and Weber used urea to eliminate the excess of hypobromite. In adapting Sakaguchi's test to histochemical use, Serra (1944, 1946) followed Weber in using hypobromite and urea. I preferred not to do so, for three reasons. (1) The validity of the test rests upon Sakaguchi's and Poller's studies, which were made with hypochlorite. (2) Hypochlorite is much more readily available than hypobromite. (3) Rapid development of colour is not necessary in non-quantitative studies.

The amount of α-naphthol solution and Milton used in the test is measured by drops. There is no point in using more accurate measures, for the exact amounts of α-naphthol and hypochlorite necessary to achieve the full reaction and leave none over depend on the amount of arginine (or other positively-reacting substance) in the section to be tested; and this will obviously vary widely in different cases.

The concentration of sodium hydroxide is kept fairly low, so as not to damage tissues.

In this test I introduce the use of pyridine as a mounting medium. It gives good definition not only in this test, but with basic dyes and dye-lakes in general. The refractive index at the D line is 1·509. Pyridine may be used alone, or chloroform (refractive index 1·449) may be mixed with it. The purpose of the chloroform is to prevent the solution of the film of celloidin that holds the section in place.

Detailed Description of the Histochemical Test

Almost any routine fixative may be used. Zenker's and Bouin's fluids, Heidenhain's 'Susa', mercuric-acetic, and formaldehyde-saline are all suitable. Zenker's fluid seems slightly preferable to the others. The following solutions are also required:

Celloidin, 1 per cent. in a mixture of absolute alcohol and ether in equal volumes.

Sodium hydroxide, 1 per cent. aqueous.

α-naphthol, 1 per cent. in 70 per cent. alcohol.

Milton (or another sodium hypochlorite solution of the same strength).

A mixture of 3 volumes of pyridine with 1 volume of chloroform.

The test is performed as follows:

I. Place a piece of tissue (preferably not more than 3 mm. thick) in one of the fixatives mentioned above. Leave for 24 hours.

II. Wash out, dehydrate, &c., as usual, and embed in soft paraffin wax.

III. Cut sections at 16μ, and attach them to slides with Mayer's glycerine and albumen.

IV. Remove the wax with xylene and wash in absolute alcohol.

V. Dip the slide in the celloidin solution. Wipe the back of it. Drain the slide and let the alcohol and ether evaporate partly (but not completely).

VI. Pass the slide through 90 per cent. and 70 per cent. alcohol to water.

If the fixative contained mercuric chloride, give the usual treatment with iodine-alcohol and sodium thiosulphate at this stage.

VII. Put 2 c.c. of the sodium hydroxide solution in a watch-glass. Add 2 drops of the α-naphthol solution, and then 4 drops of Milton. Mix the fluids by drawing them into and pressing them out of a pipette several times.

VIII. *Without waiting*, remove the slide from water, jerk the water off it (the front of the slide cannot be wiped, as a cloth would destroy the celloidin film), lay it flat, and flood it with about 1 c.c. of the mixture made at stage VII. Leave the slide for 15 minutes. The colour develops gradually and reaches its maximum in this time.

IX. Drain the fluid off the slide. Blot the slide with filter-paper and place it in a jar of the pyridine-chloroform mixture. Leave for about 3 minutes.

X. Mount in pyridine-chloroform (or in pyridine). If it is wished to keep the slide for more than an hour, it is best to paint round the edges of the coverslip with gold size.

Result. A pink or red colour indicates the presence of arginine (free or combined) or some other positively-reacting guanidine derivative.

The colour is not permanent. Full intensity is retained for several hours and the colour then fades gradually. Very strongly reacting objects still show colour after the lapse of some weeks.

Instead of flooding the slide with the mixture at stage VIII, one may advantageously use Gurr's staining plate.

Results of the Application of the Test to Tissues

Since most proteins contain arginine, protoplasm gives a positive reaction. Most of the so-called tests for proteins are so weak when applied to sections that ordinary ground-cytoplasm is often uncoloured or only slightly tinged. It is generally not very easy to demonstrate by histochemical methods one of the most fundamental facts of biology—that cytoplasm contains protein. The Sakaguchi test, as modified for histochemical use, generally gives clear evidence of the existence of arginine in ground-cytoplasm. In some cases (e.g. in the epithelial cells of the mammalian intestine, or the spermatocytes and young spermatids of the frog) the reaction is quite strong. Smooth muscle also reacts fairly strongly. The reaction is naturally much stronger in the aleurone grains of plants, on account of the higher concentration of protein. The aleurone grains in the cotyledons of the apple-seed (Fig. 1 A) give a particularly strong reaction and provide a suitable object for a first trial of the method by botanists. (The testa of the seed must, of course, be removed before fixation, and the cotyledons should be cut across to allow the fixative to enter easily.) Chromatin is usually more darkly stained than nuclear sap and cytoplasm, and nuclei therefore stand out slightly in most tissues. The nuclei of the spermatozoa of many animals react more strongly than any other cell-constituent that I have studied. I have already mentioned those of the snail in my earlier paper (1944). Those of the newt (Fig. 1 B) and salamander give a particularly powerful reaction. The testis of the newt is the most suitable

object for a first trial of the method by zoologists. The organ may be taken at any time between late summer, when spermatogenesis is complete, and the

FIG. 1 A.

FIG. 1 B.

FIG. 1. Photomicrographs of sections stained by the modification of Sakaguchi's test described in this paper. The scale drawn below each photomicrograph represents 0·1 mm.
A. Part of the cotyledon of an apple-seed.
B. Part of the testis of a common newt (*Triturus vulgaris*).

beginning of the breeding season in spring. The powerful reaction in the nuclei of spermatozoa accords with the high arginine-content of the basic proteins that occur there in combination with nucleic acid.

The staff of the Botanical Garden at Oxford kindly provided me with some seeds of *Galega officinalis*. These showed nothing remarkable when subjected to the test. No method is known for the histochemical distinction between galegine and arginine. Klein and Schlögl's method (1930) for the microchemical identification of galegine has not been adapted to histochemical use. The cells of the cotyledons showed large, strongly reacting aleurone grains, which may have contained galegine as well as combined arginine. (It is stated by Muller (1925) that the seeds of *Galega officinalis* contain no free arginine.)

SUMMARY

1. A review of known facts indicates that Sakaguchi's test gives a positive reaction with all substances having this formula (and with no others):

$$\alpha-N{=}C\begin{array}{c} \overset{\displaystyle H}{\underset{\displaystyle |}{N}}-\beta \\[4pt] \gamma \\[4pt] \underset{\displaystyle |}{N}-\underset{\displaystyle |}{C}-\delta \\[2pt] H \quad \epsilon \end{array}$$
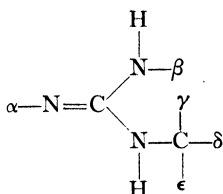
where α is H or CH_3 and β is also H or CH_3.

2. Arginine, being a constituent of most proteins, is by far the commonest positively-reacting substance that occurs in the tissues of plants and animals. Galegine and agmatine, which also react positively, are of very restricted occurrence.

3. Details are given of a method whereby Sakaguchi's test may be applied to paraffin sections.

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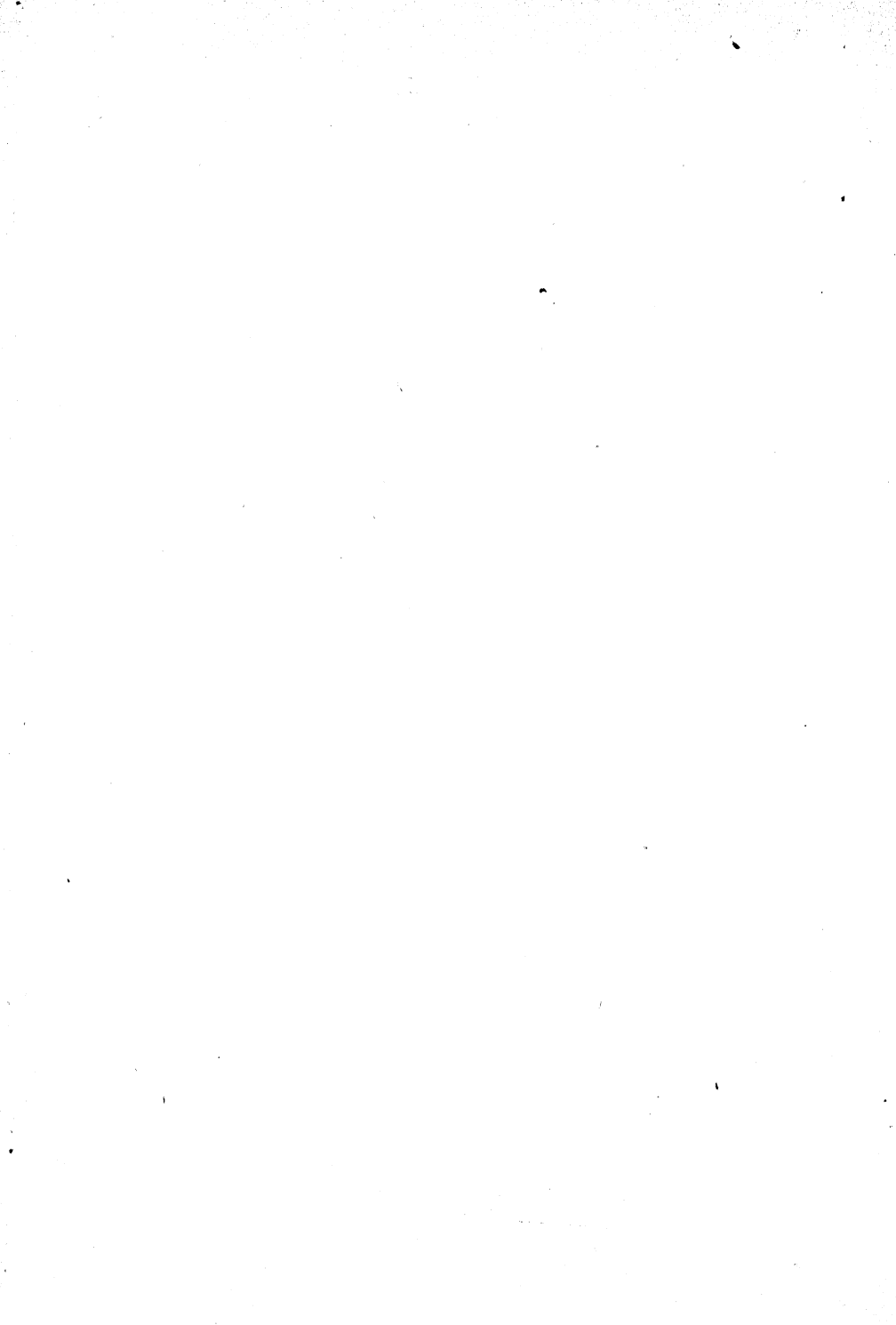
Ester Wax: A New Embedding Medium

BY

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CONTENTS

1. INTRODUCTION

THE aim of this investigation was to produce new ribboning embedding media which would, as far as possible, combine some of the advantages of paraffin wax and celloidin with a reduction of their disadvantages.

The research required the selection and manufacture of many organic products. Thanks are expressed to Dr. T. Malkin, Dr. C. E. H. Bawn, and Dr. G. T. Young of Bristol University and to Dr. E. Cockbain of the Imperial Chemical Industries for help and advice on the preparation of uncommon esters.

The research was conducted under the supervision of Professor C. M. Yonge.

2. HISTORY OF RIBBONING EMBEDDING MEDIA

Paraffin wax as a ribboning embedding medium has been known since 1882. It has been the main embedding medium ever since, but has been subject to additions and modifications at various times. Diethylene glycol distearate as a ribboning medium was first investigated by Orton and Post (1932) and later by Cutler (1935), but they abandoned the material because it had a number of weaknesses.

3. DEFINITION OF TERMS

Before proceeding to deal with the difficulties which arose in the course of this research, definitions of some of the terms employed in the technique of sectioning are necessary.

Section. This is a thin slice of material embedded in a suitable matrix. It is a single slice, cut on a microtome and may vary in thickness from 1 to 60 μ.

Section compression. Taking paraffin wax as an example, if the edge of the block at right angles to the knife is measured, and the corresponding edge of a section cut from that block is measured, it will be found that the length of the section is smaller than the side of the block. This diminution is due to compression of the embedding material as it passes the knife edge. It may amount to as much as 33 per cent. of the original breadth of the block, increasing inversely with the thickness of the section. It appears to be due to the crystalline nature of the embedding matrix, the particles of which slide upon one another with ease as compared with the particles which go to make up an amorphous material such as celloidin.

Section de-compression. This means the expansion of previously compressed material towards its original length. In actual measurement it is rare for decompression to equal compression, so that even when sections have been carefully treated on hot water there are still some traces of compression left.

Section expansion. This means an increase in either the length or breadth of a section after flattening on warm water as compared with the corresponding side of the block. It is almost unknown.

Section crumpling. This is a puckering of the section in any direction or plane.

Section curling. The section curls upon itself, a condition which may be carried to a point at which a cigar-like cylinder is produced.

Section corrugation. This is a form of crumpling in which the puckering takes place in an even series of lines parallel with the knife edge.

Section breakage. Very thick sections may break into a number of jagged bars roughly parallel with the knife edge.

Section flattening. This means the reduction of a crumpled, curled, or corrugated section to flatness, commonly by floating the section on warm water. Such flattening is usually accompanied by an increase in length, an increase which must not be confused with the increase due to de-compression. Only those sections which are flat as they leave the knife can be used for studying compression or de-compression.

Ribbon. A ribbon is a series of consecutive sections firmly joined to one another. The sections comprising it should be flat, easy to enumerate, and the ribbon easy to handle. Thus any embedding material from which ribboning is expected must show both cohesion and adhesion. There may be doubts as to the number of adhering sections which properly constitute a ribbon. It has been decided in this paper that a minimum of ten sections is a reasonable figure.

Section range (*Range of thickness of single sections*). This is an expression of the performance of a wax of stated composition or melting-point at a given room temperature. The range varies with the room temperature and is also influenced by the sharpness of the knife. It refers to single sections only.

Ribbon range (*Range of thickness of section-ribbons*). This indicates the

ribboning performance of a wax of stated composition or melting-point at a stated room temperature. It is generally a smaller range than the section range of the same wax, because the factors responsible for successful ribboning are not the same as those required for successful production of single sections. It is dependent on a wide variety of factors which include room temperature, block temperature, age of the block, knife sharpness, interference by static electricity, &c.

4. PRODUCTION OF ESTER WAX

In the production of ester wax, paraffin wax was taken as type of embedding matrix and after consideration of the technical problems involved the following list was compiled of the essential properties of any new ribboning embedding medium. The material should be:

1. Other than a hydrocarbon
2. Soluble in alcohol, dioxan, &c.
3. Suitable for sectioning and ribboning
4. Molten between 30° C. and 50° C.
5. Translucent or transparent
6. Stable
7. Homogeneous
8. Harmless to embedded material
9. Capable of being flattened after ribboning
10. Non-poisonous
11. Easy to handle
12. Cheap
13. Odourless
14. Colourless.

No natural wax was found which combined all these properties. Pure, refined waxes or fats were less suitable than mixed esters. Finally five synthetic materials were selected as possible bases:

Diethylene glycol distearate
Diethylene glycol monostearate
Glyceryl tristearate
Glyceryl monostearate
Stearin.

Of these substances diethylene glycol distearate proved to be the most satisfactory. It was used as the main constituent of a series of mixtures which were made to combat its crystallinity and to prevent air from creeping in during the making of blocks. Over 200 reagents were used, and after more than 2,000 mixtures had been tested the following was found to be satisfactory:

Diethylene glycol distearate	73 gm.
Ethyl cellulose, low viscosity	4 gm.
Stearic acid	5 gm.
Castor oil	8 gm.
Diethylene glycol monostearate	10 gm.

As a softening agent castor oil was inferior to octadecanediol diacetate, but as the latter had to be made specially it was finally abandoned.

The following are among the main physical characters of this mixture:

Melting-point	. .	48° C.
Section range	. .	2–20 μ at room temperature of 66° F.
Ribbon range	. .	2–15 μ at room temperature of 66° F.
Compression after flattening		7·6 per cent. at 10 μ.

The above mixture may be varied according to the tissue to be embedded. For hard tissues the ethyl cellulose content may be increased to 5 per cent; for very hard material up to 7 per cent., but above 7 per cent. the mixture becomes too viscous to be used. The higher the amount of ethyl cellulose present the longer is the infiltration required.

If a soft wax is required, as much as 20–30 per cent. of diethylene glycol monostearate may be used.

A description of the constituents is given below:

Diethylene glycol distearate. This is a commercial product which contains a high proportion of diethylene glycol dipalmitate. It frequently contains dust and gritty impurities which may be removed by filtering through a hot filter funnel using a rapid filter paper. A good paper is Barcham Green 904.

It is better to filter the main ingredient in this way rather than the final mixture because the viscosity of the ethyl cellulose makes filtering a slow process. Exposed to the air the ester develops a bloom, which is composed of crystals, but which does not appear to affect the inner parts. This bloom also makes its appearance on the surface of the embedding mixture.

Ethyl cellulose. This is a white, very light, powdery substance, which may be obtained in three grades of viscosity—low, medium, and high. There is little advantage in increasing the viscosity of any embedding medium and the low viscosity grade was used. A certain amount of dust is present in the commercial product, and this can be removed only by allowing it to settle in the mixture and decanting off the clear liquid. The presence of ethyl cellulose in the mixture imparts toughness to the section, enables the ribbon to be handled easily, and prevents the sections melting or coming to pieces on the water when flattening.

Stearic acid. This is the purified form of stearin, and was used in all the experiments. It assists the mixture by promoting greater homogeneity, and by improving its ribboning properties.

Castor oil. The purified, colourless product is preferred. It is the softening agent in the mixture.

Diethylene glycol monostearate. This is a soft, lard-like ester, which improves the texture of the mixture, and assists ribboning. It generally contains free diethylene glycol which may be removed by melting and decantation.

To make the mixture for embedding, first weigh out the castor oil into a porcelain pot with a handle. Then weigh out the diethylene glycol distearate and add about 15 gm. of the 73 to the castor oil. Melt over a bunsen flame and when hot add the ethyl cellulose, already weighed out. Heat until this dissolves

and then add the rest of the diethylene glycol, the stearic acid, and diethylene glycol monostearate. Allow to cool and the mixture is ready.

On being made into blocks it forms a translucent wax-like substance. If the pure wax used for making the block is heated approximately $10°–15°$ C. above its melting-point and then poured into the mould a clearer block will be produced than one in which the wax was used when just at its melting-point. The specimen should be in another container and should be put into the block when it has cooled sufficiently for a skin to form next to the metal L-pieces.

5. ESTER WAX SOLVENTS

Ester wax is designed so that the use of hardening hydrocarbons, either for clearing or embedding, may be avoided. A representative selection of clearing agents or solvents follows. The figures indicate the minimum temperature at which ester wax is soluble in all proportions with the given fluids.

TABLE I. *Temperatures at which Ester wax is soluble in pure solvents, and in aqueous dilutions*

I Wax insoluble; IM Solvent immiscible with water; S Wax soluble at all temperatures; Results in degrees Centigrade.

Solvent	Percentage of water				
	0	10	20	30	40
Alcohols					
Methyl	I	..	..	..	..
Ethyl	43	I	..	..	..
iso-Propyl	38	45	50	I	..
n-Butyl	38	IM	..	..	..
Amyl	36	IM	..	..	..
Octyl	33	IM	..	..	..
Methylated spirits	43	I	..	..	..
Ethylene glycol	I	..	..	..	..
Propylene glycol	I	..	..	..	..
Butylene glycol	I	..	..	..	..
Terpineol	22	IM	..	..	..
Ethers					
Ethyl ether	S	IM	..	..	..
Ethylene glycol mono-ethyl ether	38	65	I	..	..
Ethylene glycol mono-methyl ether	65	I	..	..	..
Ethylene glycol mono-butyl ether	34	40	44	45	I
Diethylene glycol	I	..	..	..	..
Diethylene glycol mono-ethyl ether	I	..	..	..	..
Diethylene glycol mono-butyl ether	38	45	48	I	..
Dioxan	S	46	I	..	..
Esters					
Methyl acetate	34	IM	..	..	..
Ethyl acetate	S	IM	..	..	..
iso-Propyl acetate	S	IM	..	..	..
n-Butyl acetate	S	IM	..	..	..
Amyl acetate	S	IM	..	..	..
Ethylene glycol diacetate	I	..	..	..	..

TABLE I (contd.)

Solvent	Percentage of water				
	0	10	20	30	40
Esters (cont.)					
Glyceryl mono-, di-, and tri-ace-tates	I	..	..	..	..
Ethyl butyrate . . .	S	IM	..	..	..
Ethyl lactate	40	75	I	..	..
Butyl lactate	33	IM	..	..	..
Amyl lactate	32	IM	..	..	..
Benzyl benzoate . . .	33	IM	..	..	..
Ethylene glycol mono-ethyl ether mono-acetate . . .	33	IM	..	..	..
Hydrocarbons					
Xylene	S	IM	..	..	..
Benzene	S	IM	..	..	..
Toluene	S	IM	..	..	..
Ligroin	S	IM	..	..	..
Chlorinated hydrocarbons					
Chloroform	S	IM	..	..	..
Carbon tetrachloride . .	S	IM	..	..	..
Pentachlorethane . . .	S	IM	..	..	..
Ethylene dichloride . .	S	IM	..	..	..
Ethylene chlorhydrin . .	S	IM	..	..	..
Ketones					
Acetone	33	I	..	..	..
Diacetone alcohol . . .	40	I	..	..	..
Cyclohexanone . . .	20	I	..	..	..
Amines					
Anilin oil	18	I	..	..	..
Aldehydes					
Cinnamaldehyde . . .	33	IM	..	..	..
Anisaldehyde	33	IM	..	..	..
Salicylaldehyde . . .	25	IM	..	..	..
Natural oils					
Arachis . . .	S	IM	..	..	..
Castor	S	IM	..	..	..
Cedarwood	S	IM	..	..	..
Clove	S	IM	..	..	..
Olive	S	IM	..	..	..
Origanum	S	IM	..	..	..
Peppermint	S	IM	..	..	..
Wintergreen	S	IM	..	..	..

From the figures given in Table 1 it is clear that ester wax is soluble in a wide range of substances, any of which could be used as clearing agents. The following are suggested:

Ethylene glycol mono-ethyl ether (Cellosolve).
Ethylene glycol mono-butyl ether.
Diethylene glycol mono-butyl ether.
Dioxan.
Cedarwood oil.

6. INFILTRATION WITH ESTER WAX

As with paraffin wax specimens may be taken directly from the wax solvent into the molten wax. This is not recommended. The more gradual change effected by interposing a solution of ester wax in the solvent before finally placing in molten wax is preferable.

The correct time during which a specimen should remain in molten ester wax depends on the size of the specimen, but a few representative examples are given below.

Frog egg, 2·5 mm. diameter	3 hours
Pieces of *Lumbricus*, 15 × 15 mm. (diam.)	15 hours
Pyloric stomach, Cat, 15 × 15 × 15 mm.	24 hours
Slice of brain, Cat, 34 × 25 × 10 mm.	60 hours

In all cases it is advisable to change the specimen from one bath of molten wax to a second and a third before finally making the block. This is particularly important when using clearing agents of low evaporation rates, e.g. dioxan.

The presence of free stearic acid in the mixture leads to the production of copper stearate should copper pans be used. This does not appear to affect the tissue, but a piece of paper placed at the bottom of the copper pot containing the molten wax will prevent contact with the copper stearate. Porcelain pots are recommended, however.

The times taken for producing stained serial sections of *Hydra oligactis* may be of interest.

Fixed Carnoy	5 min.	Staining and flattening	5 min.
Washed, dehydrated, and		Drying	10 ,,
cleared in 'Cellosolve'	3 ,,	Wax removal, counterstain-	
Infiltrated, 2 baths	5 ,,	ing and mounting	7 ,,
Block-making and cooling	10 ,,		
Ribboning, 6 μ	15 ,,	Total	one hour.

7. BLOCK-MAKING WITH ESTER WAX

The well-marked crystalline nature of diethylene glycol distearate makes block-making with ester wax a careful operation. Air infiltration is the chief difficulty. The following procedure, using metal L-pieces, was found most useful.

Adjust the L-pieces to the size of the block required on a small piece of glass such as a 3 × 1 in. slide. Pour the molten ester wax into them. Take the specimen from molten wax in the embedding oven and place it in the wax in the L-pieces so that it lies on the skin of congealed wax next to the glass slide, and in the angle of one of the L-pieces rather than in the centre. Place the slide with the L-pieces on it in a Petri-dish of cold water, so that the block is

not submerged, and as cooling and contraction of the wax takes place fill in the hole which forms either by using a hot spatula or needle or by dropping molten wax into it. A slight amount of air infiltration will take place at the top of the block, but this is usually so far away from the specimen that it gives no trouble and is removed when the block is trimmed.

The quicker a block cools the better will be its texture. It is always preferable to have the L-pieces cold before pouring molten wax between them, because a skin of wax is quickly produced next to the cold metal. As contraction of the wax takes place it can shrink away from the sides of the mould, and in this way the centre of the block may contract less. A better block is produced if the wax used in making it is heated over a bunsen to about 20° C. above its melting-point just before pouring into the L-pieces.

8. Cutting Ester Wax Sections

All the precautions to be taken when cutting sections of paraffin wax must be observed when cutting sections of ester wax. In addition sections of ester wax must be cut *very slowly*, otherwise badly crumpled sections will be produced.

It is in the cutting of ester wax sections that one of the fundamental differences from paraffin wax may be observed. Paraffin wax is made up of a number of minute crystals and as the block passes the knife edge the crystals can be felt grating upon it. In addition the movement of the crystals produces a shortening, i.e. compression, of the section. This compression, even though followed by de-compression when the section is placed on warm water, is certainly harmful to embedded tissues. The smooth appearance of celloidin sections is considered due not only to the avoidance of heat in the technique but also to the fact that celloidin is not crystalline and that compression is practically non-existent.

Ester wax closely resembles celloidin in this respect. Its very large crystals adhere to one another so firmly in the properly cooled condition that it behaves as an amorphous mass even though the crystals are visible under the microscope and are much larger than those of paraffin wax. The size of a slowly cooled crystal may be about 4 mm. in diameter; the commonest size is 2–3 mm.

A sharp microtome knife is essential, and it should be stout and securely held. Ester wax is a very hard material compared with paraffin wax and a microtome knife which will not bend away from a block of paraffin wax during cutting will frequently do so when ester wax is used. Even a trace of looseness in the bearings of sliding parts of the microtome will lead to sections of unequal thickness, and this will produce poor ribboning or no ribboning at all.

For thick sections the material may be cut on the microtome the day the block is made, but thin sections of 3–6 μ are better obtained on the second or third day.

For very thick sections and for block trimming it is an advantage to place the block in a warm oven or in warm water for a minute or two.

9. Flattening Sections of Material Embedded in Ester Wax

It has already been stated that one of the best embedding mixtures is:

Diethylene glycol distearate	73 gm.
Ethyl cellulose, low viscosity	4 gm.
Stearic acid	5 gm.
Castor oil	8 gm.
Diethylene glycol monostearate	10 gm.

The object of this research was to produce a wax which was not a hydrocarbon and which had a greater water tolerance than paraffin wax. The above formula and the section on ester wax solvents shows that this has been done. But increased water tolerance in an embedding medium also implies a greater affinity for water by the sections, and also a differential affinity, because the tissue in the section is much nearer its natural condition than when embedded in a hydrophobic wax and during flattening on warm water takes up more water than the wax. This differential intake leads to crumpling of the section so that when it is finally dried after flattening on water small folds are present.

This was the case with material sectioned in the mixture given above. The sections, both wax and specimen, were flat when on warm water on a slide, but crumpled when the slide cooled later on. The possibility that successful flattening of the specimen might be induced by lowering the surface tension of the flattening water, by introducing substitutes for stearic acid in the formula, and by adding a water-soluble wax solvent to the flattening water was therefore investigated.

Reagents such as sulphonated castor oil and sodium taurocholate were used for lowering the surface tension. Stearic acid esters were substituted for stearic acid in the formula. Cellosolve and ethyl lactate were included in the water-soluble wax softeners. Variations in the pH of the flattening water were also tried.

The results showed that no beneficial effects followed from the use of such reagents, and further experiments were abandoned.

A noticeable feature of ester wax is the tendency for individual sections of a ribbon to separate during flattening. This frequent fault was considered as being due to a low margin of ribboning power. Such a margin could possibly be increased by further experiments with crystalline materials such as stearic acid, but even then the greater the amount of adhesive material added the more would the special properties of diethylene glycol distearate be reduced. Accordingly no further experiments were made in this direction.

Considerable improvement in flattening was noticed when the sections were placed in an oven at 35°–40° C. after flattening on water in the usual way. In this manner they dried at the temperature at which they were flattened and were not allowed to become cold and wrinkled. It is essential that ester wax sections should be dried in this manner, and that when placed in the oven there should be sufficient water beneath the ribbon to act as a lubricant.

10. STAINING SECTIONS OF MATERIAL EMBEDDED IN ESTER WAX

Because of its greater water tolerance it is possible to stain sections of material cut in ester wax in two ways:

A. In the ribbon, i.e. with the wax still present;

B. After removal of the wax, as with paraffin wax sections.

The second method is well known and needs no description. The steps of the first method are as follows:

1. Smear a microscope slide with albumen in the usual way.
2. Flood the slide with a solution of methylene blue in water (0·001 per cent. or stronger). Place the ribbons on the solution and flatten by warming.
3. Drain away the excess staining solution and then wash the slide with the section still on it by adding a few drops of water and draining again. Repeat this until no more blue comes away.
4. Leave sufficient water on the slide to float the ribbons and place in an oven at 45°–50° C. to complete the flattening and to dry. Twenty minutes to one hour is enough. Any of the nuclear coal-tar dyes may be used in this way.

For removing the wax and counterstaining the following solutions are necessary:

	1	2	3	4	5	6	7	8
Cellosolve (Ethylene glycol mono-ethyl ether) .	..	..	10	20	40	80	100	100
Ethyl acetate . . .	..	..	45	40	30	10	..	..
Xylene	100	100	45	40	30	30	..	..

Erythrosin or eosin may be used as a counterstain dissolved to saturation in any solution from No. 4 to No. 7. The higher the percentage of cellosolve present the more will the methylene blue be extracted. The ordinary procedure is outlined below:

1. Partly remove wax from the sections with xylene, 2–5 minutes.
2. Continue this process in the 10 per cent. cellosolve mixture.
3. Differentiate the methylene blue in mixture No. 6 or No. 7.
4. Counterstain in No. 8, to which has been added eosin or erythrosin to saturation. If it is found that the methylene blue is extracted, the lower percentage cellosolve solutions with eosin to saturation should be employed instead.
5. Transfer the slide from No. 8 to No. 4, bringing a good supply of stain on the slide. This stain will then be forced into the material owing to the decreased solvency of the No. 4 mixture. Rinse well to remove excess stain solution.
6. No. 3 solution for 2–4 minutes.
7. Xylene (two baths).
8. Mount.

11. Mounting Stained Ester Wax Sections

After counterstaining, sections are placed in pure xylene and mounted in the usual way. Canada balsam in xylene is not recommended as methylene blue invariably fades in this medium. The proprietary product 'Sira' and the polystyrene mounting medium put forward by Kirkpatrick and Lendrum (1941) do not make methylene blue preparations fade. They are recommended in place of balsam in xylene.

Stained preparations should be kept out of direct sunlight or very bright daylight.

12. Summary

Ester wax is a new ribboning embedding medium. It consists of five substances which may be varied in proportion so that media of different characteristics may be produced. A mixture which will meet most requirements is as follows:

Diethylene glycol distearate	73 gm.
Ethyl cellulose	4 gm.
Stearic acid	5 gm.
Castor oil	8 gm.
Diethylene glycol monostearate	10 gm.

The following facts relate to this mixture:

Melting-point. 46–8° C.

Section range. 2–20μ at a room temperature of 64° F.

Ribbon range. 2–15μ at a room temperature of 64° F.

Final compression loss. 7·6 per cent. at 10μ (after flattening).

Solvents. Alcohols, ethers, esters, ketones, hydrocarbons, aldehydes, chlorinated hydrocarbons, natural oils, &c.

Cutting. This must be done more slowly than with paraffin wax.

Flattening. Sections may be flattened on tap-water or aqueous staining solutions. Flattening must be completed in a drying oven at 40–5° C. with sufficient water under the sections to float them.

Staining. Two methods: (1) In the ribbon; (2) As for paraffin wax sections.

Pre-mounting fluid. Xylene, benzene, ligroin, &c.

Mounting fluid. 'Sira', or Kirkpatrick and Lendrum's polystyrene mounting medium.

Conclusion. It is believed that ester wax may open up new methods in histology and cytology. Its greater water tolerance and its property of ribbon staining followed by drying constitute an advantage found in no other embedding medium. Greater use of the wide range of solvents as stain carriers is indicated.

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Experimental Data on the Function of the Interstitium of the Gonads: Experiments with Cockerels

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With one Plate and seven Text-figures

CONTENTS

1. INTRODUCTION

THE problem of the localization of the endocrine function of the testes in birds as well as in mammals is still unsolved, notwithstanding the large numbers of papers relating to it. The results of our experiments with young mice, forming the first part of these investigations, have been published by one of us (Sluiter, 1945).

It is generally known that the hypothesis of Bouin and Ancel (1903), who claimed that the testis hormone is formed in the so-called interstitial cells, was violently opposed by Stieve (1921a, 1921b, 1923, 1926); later investigations, however, especially those of Benoit (1924a, 1924b, 1929) pointed out that the endocrine function of the cock's testis is not interfered with when the generative tissue is totally eliminated by Röntgen-radiation. Hence we must assume *per exclusionem* that in the avian testis the male hormone is formed somewhere in the intertubular tissue. Benoit (1929) ascribed this function in the cock's testis to intertubular cells, which morphologically resemble gland cells. He identifies them with the well-known so-called Leydig cells, which according to him possess a glandular appearance periodically. Stieve on the contrary does not find any secretory cells in the intertubular tissue. According to him the fully developed Leydig cells, large conspicuous cells containing many lipoid granules, have only a trophic function.

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In the present paper we will try to answer the following questions:

1. Is it possible to locate, in the intertubular tissue of the cock's testis, cells that may be regarded as glandular on the evidence of their cytological structure and the chemical nature of their contents? (Cf. p. 144, under 2.)

2. What is the physiological significance of the intracellular storage of lipoids in the intertubular tissue? (Cf. p. 146 under Conclusion 3.)

3. Are the number, the functional changes of structure, and the micro-chemical reactions of the contents of the interstitial cells such that these may be regarded as the cells that produce the male sex hormone; and are this number and these structural changes present at an appropriate period of life? (Cf. p. 148 under Conclusion 4.)

We have attempted to answer these questions by a careful cytological investigation of the different intertubular cell-types, and of their changes after the administration of gonadotrophins to cockerels, which manifest their effects by an accelerated development of the head appendages.

2. MATERIAL AND METHODS

The material consisted of 31 cockerels, the ages of which varied between 2 and 200 days. In 5, being 14–52 days old, gestyl, a gonadotrophin prepared from pregnant mare serum, which has a distinct accelerating influence on the development of the head appendages, was administered. Our thanks are due to the Directors of Organon N.V., for providing us with this preparation. Each experimental bird received 4 doses totalling 100 I.U. every other day and was killed for autopsy 2 days after the last injection. Then the testes were fixed and the size of the head appendages measured. Owing to war-time circumstances we did not have the necessary photographic material at our disposal; we therefore had to use translucent paper on which images of the head appendages were projected. To establish the relative surface area of these appendages, these images were redrawn on cardboard, cut out, and weighed.

Parts of the same testis were fixed in Bouin's fluid for general staining; in Champy's fluid for staining cytological details such as mitochondria and granules with the aid of Altmann's acid fuchsin and brilliant-cresylblue; in Giroud's and Leblond's fluid for demonstrating vitamin C; in Schultz's fluid to establish the presence of cholesterol; and finally in formalin for the staining of lipoids with Sudan III. The thickness of the sections was $2-3\mu$ after Champy-fixation and 10μ in all other cases.

3. GROWTH OF THE TESTIS, OF ITS COMPONENTS, AND OF THE HEAD APPENDAGES

In cockerels' testes, besides intertubular connective tissue cells, nervous tissue, blood-vessels, and lymph-vessels, cells are found, the contents of which stain deeply with acid fuchsin after fixation in special fluids, e.g. Champy's fluid. They will be termed interstitial cells (in a restricted sense) and agree

with the large lipoid-storing Leydig cells, mentioned by Stieve, as well as with the glandular interstitial cells described by Benoit.

In Text-fig. 1 the sizes of the testes, of their components, and of the head-appendages of cockerels of different ages, some of them after gestyl-administration, are delineated. The average size of the testes is expressed by rectangles of which the length of the short and long side are proportional, respectively, to the average length of the short and long axes of both ellipsoid testes. In every rectangle the following testis-components are represented at the same magnification: testis-tubules (white), interstitial cells (black), and rest of the intertubular tissue (dotted). In normally developing cockerels, respectively 14, 38, 66, and 200 days old (Text-fig. 1, a, b, c, and d), the intertubular tissue is originally larger than the testis-tubules (Text-fig. 1, a and b). Later on, the latter are relatively more developed than the intertubular tissue (Text-fig. 1, c and d). The same applies to the size of all the interstitial cells taken together, compared with that of the testis-tubules. After gestyl-administration the testes of very young cockerels increase in size (cf. Text-fig. 1, a and a_1) contrary to those of older ones (cf. Text-fig. 1, b and b_1). In both cases a marked increase of the total quantity of interstitial cells is evident, an increase which runs parallel to the enlargement of the head appendages. This fact, already known in mammals, supports the hypothesis that the male sex hormone is formed in the interstitial cells (Bourg, 1930; Sluiter, 1945).

The two-dimensional drawings of Text-fig. 1 are not adequate for a comparison of the volumes of the three-dimensional testes and their components. This comparison, however, is made in Text-fig. 2. In this graph the age of the cockerels is plotted against the relative volumes of the testes and their components. These volumes have been calculated by cutting the testis-components out of the rectangular drawings, mentioned above, weighing them, and multiplying the numbers obtained by a factor, which is proportional to the average lengths of the short axes of both testes. From this graph it follows that the development of the head appendages runs about parallel with the testis-enlargement, which for the greater part must be ascribed to a growth of the testis-tubules. The distance between the curves for the growth of the testis and of the testis-tubules may serve as an indicator for the growth of the total intertubular tissue; relatively, i.e. with regard to the whole testis, this tissue decreases in size (cf. also Text-fig. 1), but absolutely it increases considerably. This enlargement is distinctly shown by the upper broken line of Text-fig. 2, which relates to the volume of the intertubular tissue, multiplied 10 times. The fact that the intertubular tissue increases in volume absolutely and that this increase runs parallel to the development of the head appendages, had already been mentioned by Benoit (1922). This fact was overlooked and sometimes even denied by investigators, who did not think it necessary to measure the quantity of intertubular tissue by a reliable method and only estimated it.

It is customary to attach much value to the volume of the total intertubular tissue in relation to the endocrine function of the testis. Benoit (1929), however, points out that only the quantity of interstitial cells is of primary
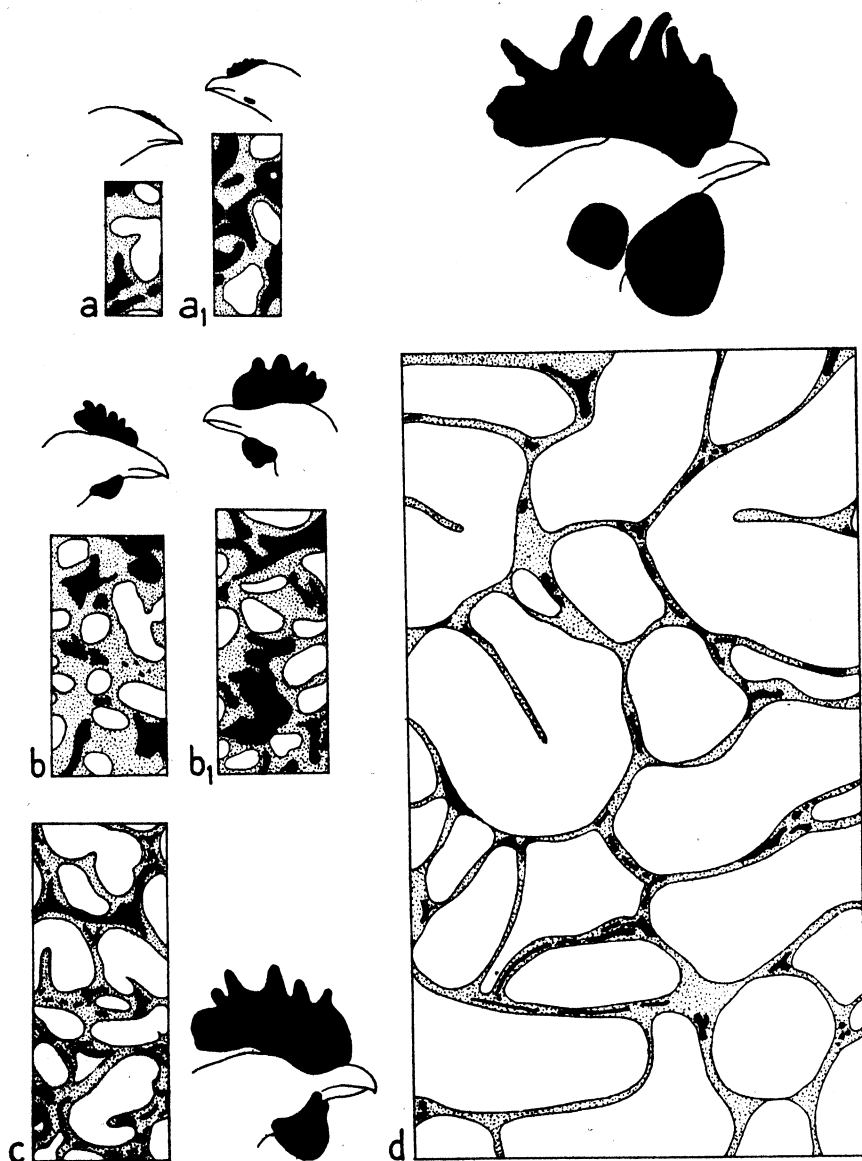
TEXT-FIG. I. Sections of testes of normal cockerels, respectively 14, 38, 66, and 200 days old (*a–d*), and of gestyl-treated cockerels, 14 and 38 days old (*a₁*, *b₁*). The sides of each rectangle are proportional to the mean sizes of the long and short axes of both testes. Champy-fixation, Altmann's acid fuchsin-staining. × 75. Heads reduced to about ⅜.
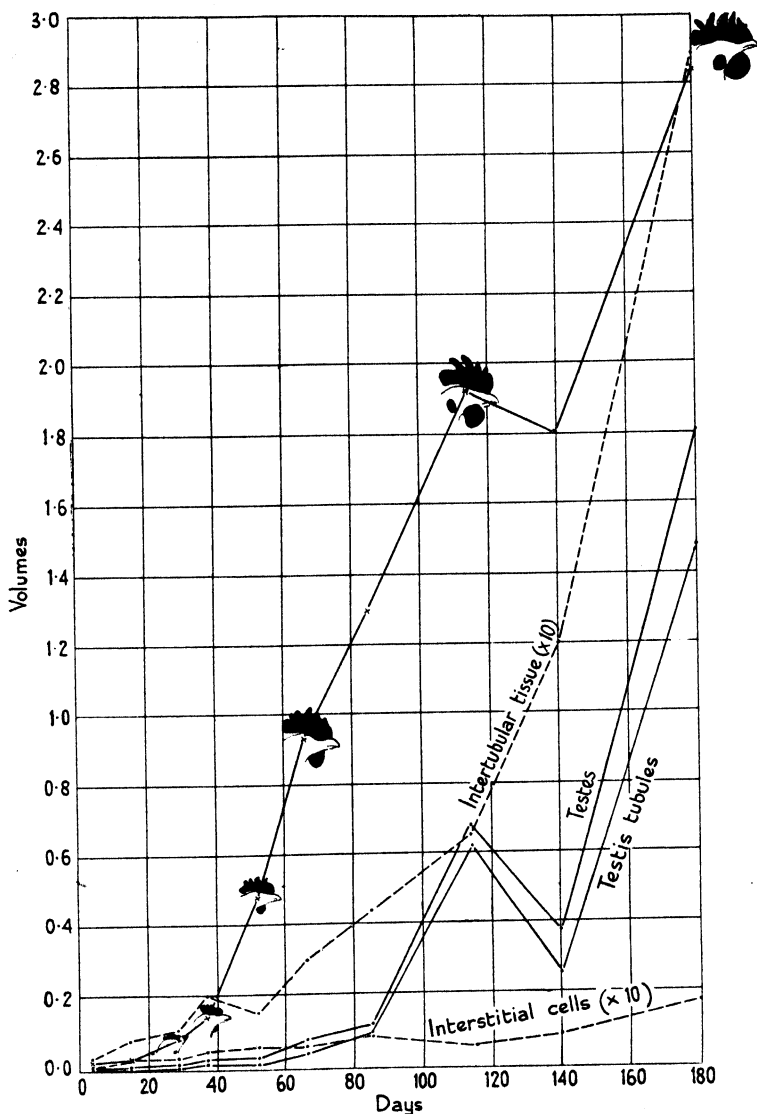
TEXT-FIG. 2. Graph showing growth of the testes, of their components, and of the head appendages in normal cockerels. Heads reduced to about $\frac{1}{11}$.

importance, because the cells which produce the male sex hormone belong to them. We entirely agree with his view. In Text-fig. 2 (lower broken line) it is shown that the volume of all the interstitial cells taken together increases slightly but distinctly and parallel to the development of the head appendages.

Summarizing, we have ascertained that parallel to the development of the head appendages the volume of the intertubular tissue as well as the total volume of the interstitial cells increases. This fact points to the endocrine function of these cells, but is no proof of it. To demonstrate the endocrine function of the interstitial cells, it is necessary to carry out an accurate quantitative investigation of the cytological structure of these cells and the functional changes which take place in them.

4. CYTOLOGY OF THE INTERSTITIAL CELLS

As already mentioned (p. 136) it does not suffice to pay attention only to size, form, or lipoid content to distinguish the interstitial cells from other cell-types in the intertubular tissue; mitochondria, granules, and vacuoles must also be considered. Therefore the structure and possible function of the intertubular cells can only be satisfactorily studied after specific fixation and staining of the testis. The laboratory routine technique (e.g. fixation in Bouin's fluid and staining with haematoxylin) is not sufficient.

According to Benoit (1929) the interstitial cells of male chicken embryos acquire a 'chondriome plus riche' towards the end of the incubation period; then also fuchsinophil granules and vacuoles make their appearance. The vacuoles, which gradually become so numerous that they fill up the whole body of the cell, possess a lipoid content. The interstitial cells remain in this stage during the first two or three months after hatching. At the beginning of the pre-spermatogenesis stage distinct changes take place in the interstitial cells; the lipoid vacuoles disappear and large quantities of 'chondriocontes', mitochondria, and fuchsinophil granules replace them. Then, according to Benoit, these cells possess their definite structure, which they retain during sexual life.

Benoit maintains that this points to the fact that the endocrine function of the testis is located in these cells. Actually the large number of fuchsinophil elements (mitochondria, granules) points to a certain activity of these cells and it is likely that this activity is a glandular one. The presence of lipoid vacuoles is not so clear, because they sometimes occur in such quantities that there is no room for a glandular function in the cell. Stieve (1923, 1926) has already remarked that the number of these lipoid-containing cells may increase enormously under certain circumstances in adult cocks, without an increased hormone production.

Consequently several questions arise:

1. Is it possible to establish, besides the presence of the fuchsinophil elements described by Benoit (1929), other cytological properties, which point to a glandular function of certain interstitial cells? (Cf. p. 144, under 2.)

2. Does any relation exist between the lipoid occurring in the interstitial cells of cockerel-testes and the endocrine function of the male gonads? (Cf. p. 146, Conclusion 3.)

3. Is it possible to establish a well-founded quantitative relation between the activity of interstitial cells and the amount of the male sex hormone? (Cf. p. 146, Conclusion 4.)

In order to answer these questions it is necessary to begin with a detailed description of the cytological structure and chemical constitution of these cells before and during the development of the head appendages and then to establish the changes occurring in these cells quantitatively, if possible by counting the cells in different functional stages.

In the first month after the cockerels are hatched, two distinctly different types of interstitial cells have been found by us in the testis (Text-fig. 3).

The *first type* relates to cells, which, after having been fixed in Champy's fluid, are almost totally filled with small vacuoles (Text-fig. $3b$), whereas some mitochondria or other very small fuchsinophil elements are found among these vacuoles. After having been stained with Sudan III (Text-fig. $3b_1$) several cells full of red globules are visible, which are apparently identical with the vacuoles shown in Text-fig. $3b$. It is certain that these cells are Leydig cells, which, being totally filled with fat, are storage-cells according to Stieve. We agree that in these cells lipoid is accumulated, but believe that Stieve goes too far when he concludes from its presence in these cells after Sudan-staining that this lipoid is used for the metabolism of the testis-tubules only. Therefore we have submitted these cells to Schultz's cholesterol-test. Text-fig. $3a$ shows that the blue oxycholesterol granules are only present in the intertubular spaces; on comparing Text-fig. $3a$ and Text-fig. $3a_1$—which is drawn from a Sudan-stained section—it is clear that the sudanophil substance consists at least partly of cholesterol or its derivatives. As cholesterol is the substance from which the synthetic androgens are derived, it is not impossible that the presence of a sudanophil substance in the intertubular tissue of the cockerel's testis may be connected with the growth of the testis-tubules as well as with the endocrine function of the interstitial cells. Finally we can conclude from Text-fig $3b_1$ that the sudanophil substance is found partly outside the interstitial cells, i.e. in the connective tissue cells surrounding them.

The *second cell-type* has quite another appearance (Text-fig. $3c$). Vacuoles are lacking in these cells, which contain many, mostly granular, mitochondria. After Sudan-staining no (or very few) lipoid globules are visible (Text-fig. $3c_1$); the mitochondria are more or less sudanophil and some lipoid globules occur in the surrounding connective tissue. According to Benoit these cells are glandular cells with a marked endocrine activity.

In cockerels, *1–2 months of age*, a *third type of interstitial cell* appears (Pl. 1, *C* and *c*), characterized by the presence of one vacuole, often so large that it occupies almost the whole cell-body (Pl. 1, *C*). Mitochondria are present in rather large numbers. After Sudan-staining (Pl. 1, c_1) these vacuoles appear to possess no sudanophil contents. These cells,
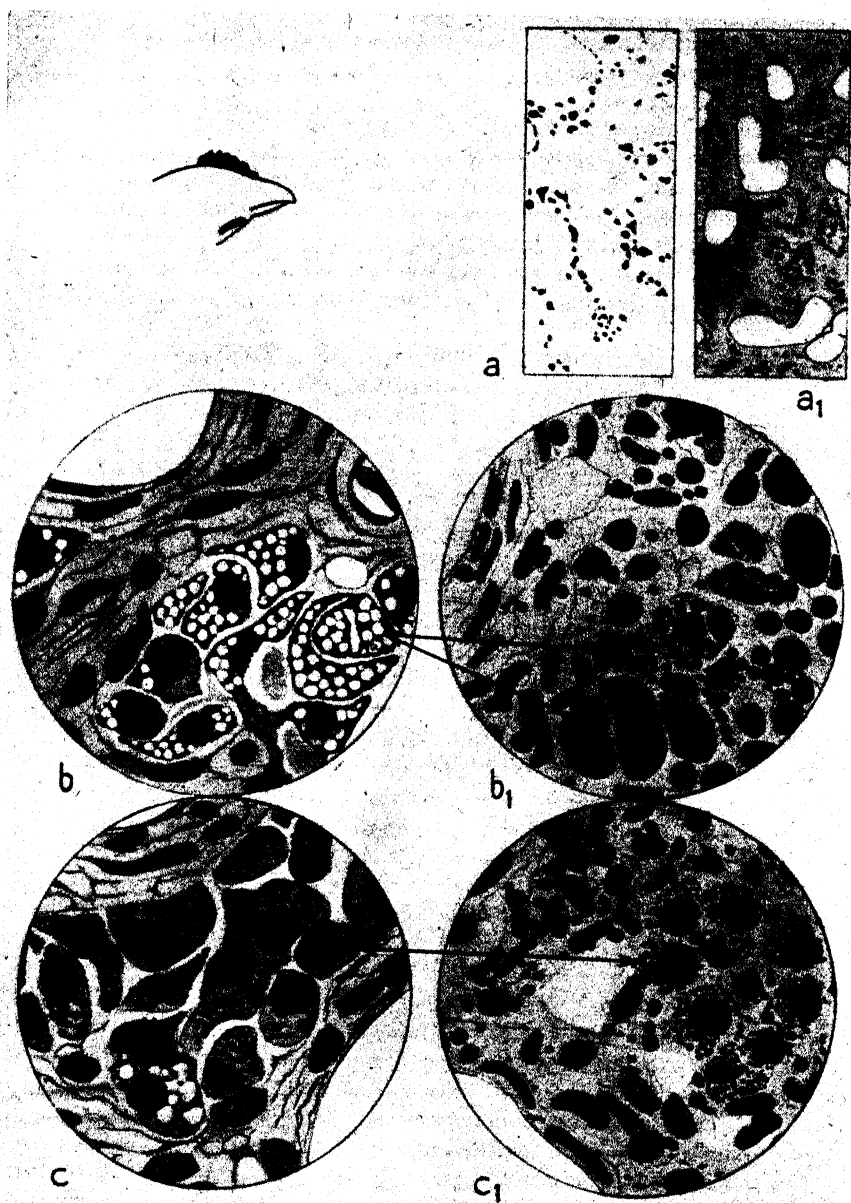
TEXT-FIG. 3. Intertubular testis tissue of cockerels in the first month after hatching. *a* and *a₁*, respectively after Schultz's cholesterol-test and Sudan III-staining (× 100); *b* and *c*, after Champy-fixation and Altmann-staining (× 1000); *b₁* and *c₁*, after Sudan III-staining (× 1000). Arrows refer to comparable interstitial cells. Head reduced to about ⅓.

which are also strongly glandular in appearance, have apparently been over-looked by Benoit.

Using Schultz's cholesterol-test we found that the intertubular sudanophil substance in these older cockerels also mainly consists of cholesterol-derivatives (cf. Pl. 1, *a* and a_1). In several places in such a preparation
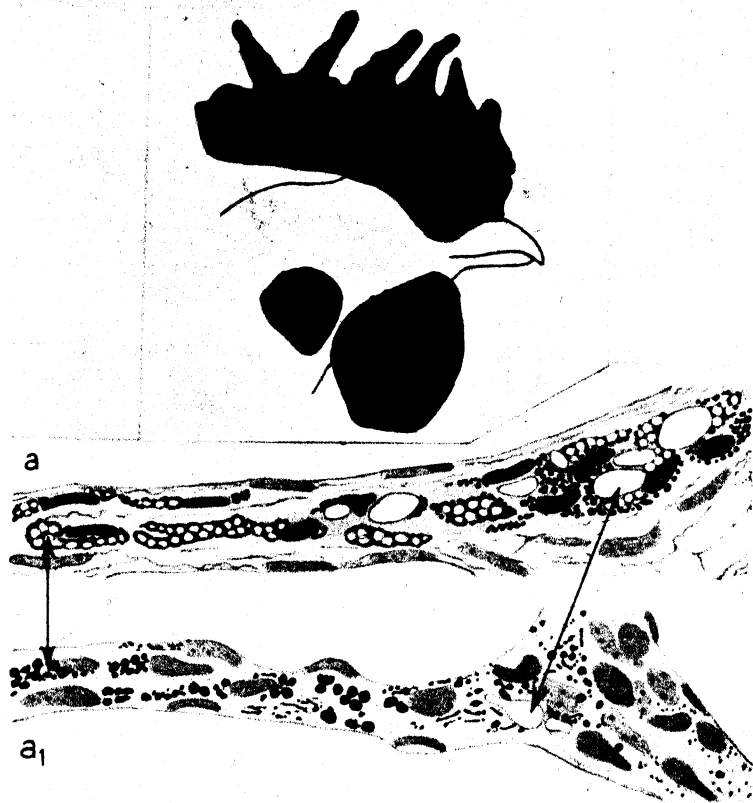
Text-fig. 4. Intertubular testis tissue of a cockerel, 200 days old. *a*, after Champy-fixation and Altmann-staining; a_1, after Sudan III-staining. $\times$ 1,000. Head reduced to about $\frac{1}{2}$.

it was even possible to identify cell-nuclei and to state that the cholesterol-derivatives were lying in the cytoplasm around the nucleus (Pl. 1, a_2). We could not establish to which of the types of interstitial cells, described above, these cells belonged.

In testes of cockerels, *2–5 months of age*, all three cell-types are present. With increasing age of the cockerels small vacuoles appear in the cells of the third type in addition to the characteristic large vacuole (Text-fig. 4*a*); they possess sudanophil contents (Text-fig. 4a_1). Moreover, the interstitial cells

of the testes of these older cockerels are smaller and more oblong than those of younger birds. This is easy to understand, as the intertubular spaces become narrower (Text-figs. 1 and 2), whereas the number of interstitial cells increases considerably.

According to Tonutti (1943) and others, the mammalian interstitial cell contains many vitamin C granules. In the testes of our cockerels the vitamin C reaction was very weak and if present vitamin C granules could be found evenly scattered over all kinds of testis tissue.
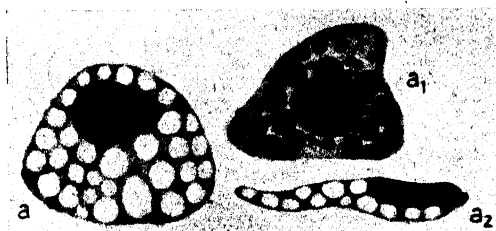
TEXT-FIG. 5. Lipoid cells from the intertubular testis tissue. Champy-fixation and Altmann-staining. ×2,250.

Summarizing, we can divide the interstitial cells into two essentially different types:

1. *Lipoid cells* (Text-fig. 5), characterized by sudanophil globules, which after Champy-fixation appear as empty vacuoles (Text-figs. $5a$ and $5a_2$). In fresh Champy-preparations these vacuoles possess a black, i.e. an osmiophil content (Text-fig. $5a_1$), which, however, dissolves after some hours in Canadabalsam. It must be emphasized that by 'lipoids' are understood several chemically diverse fatty substances and also substances which are decidedly not fats; cholesterol and its derivatives also stain with Sudan III.

2. *Secretory cells*, characterized by the presence of numerous, mostly granular (Text-figs. $6a$, $6b$, $6c$) and sometimes filamentous (Text-fig. $6a_1$ and $6a_2$) mitochondria and the absence of lipoid vacuoles. These secretory cells are present in two cell-types: *secretory cells A* without (Text-figs. $6a$, $6a_1$, $6a_2$) and *secretory cells B* with one vacuole (Text-figs. $6b$, $6c$, $6d$). The contents of these vacuoles are not sudanophil nor osmiophil. After Champy-fixation the vacuole is mostly empty, but sometimes it possesses a light-refracting crystalline body (Text-fig. $6c$). Whether this corpuscle is identical with the globuline-crystalloid of Reinke, which has been described for human and other mammalian interstitial cells, could not be ascertained.

As to the mutual relation of these cell-types, it is obvious that secretory celltype B develops from type A, in which a vacuole, gradually increasing in size, appears (Text-figs. $6b$, $6c$, $6d$). According to Benoit (1927b) type A is derived from lipoid cells which lose their lipoid contents and in which more and more mitochondria develop. Though we have also observed cells which

possess a rather large quantity of mitochondria in addition to many lipoid vacuoles, we have never seen a complete series of transitional stages between lipoid cells and secretory cells A. Therefore we do not think that Benoit's hypothesis is proved.
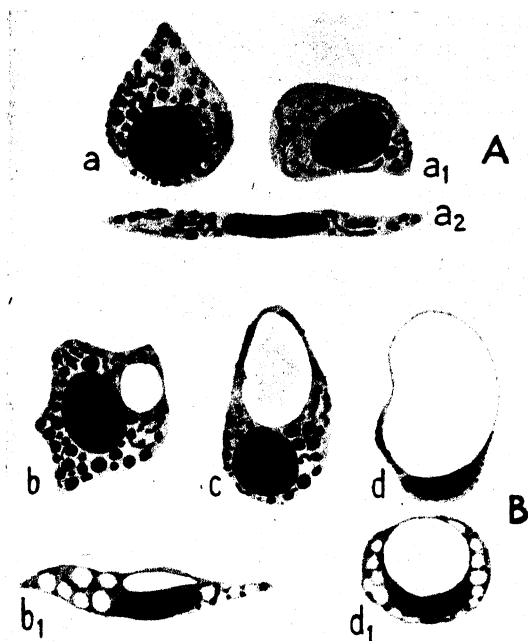
TEXT-FIG. 6. Secretory cells. *a*, *a₁*, and *a₂*, secretory cells A; *b*, *c*, *d*, *b₁*, and *d₁*, secretory cells B. Champy-fixation and Altmann-staining. ×2,250.

5. THE NUMBER OF THE INTERSTITIAL CELLS IN RELATION TO THE SIZE OF THE HEAD APPENDAGES

From the above it follows that there are interstitial cells which may be considered glandular cells. However, this does not imply that they secrete the male sex hormone. Moreover, it is necessary to ask whether the lipoid cells play a part in producing this hormone.

In order to answer these questions we have tried to find a relation between the numbers in which the three types of interstitial cells occur, and the size of the appendages, by counting these cells in the testes of normal and gestyl-treated cockerels of different ages.

In the diagrams of Text-fig. 7 the results of these counts are shown. The numbers of the three types of interstitial cells are represented by the areas of the columns; the lightly dotted areas relate to the lipoid cells; the heavily dotted areas to the secretory cells A, and the black areas to the secretory

cells B. The ordinates represent the percentages in which these cells occur. The relative surface areas of the head appendages (comb, wattles, and ear-lobes together) are shown by the numbers given above drawings to scale of each cockerel's head.

In the upper row (Text-fig. 7a) the columns relate to 23 normally growing cockerels; in the lower row (Text-fig. 7b) pairs of columns are shown, of which the left relates to a normal control bird, whereas the right one has reference to a cockerel which has been treated with gestyl.

The data on which the columns of Text-fig. 7 are based were determined in the following way. In rectangles, similar to those described on p. 137 (cf. also Text-fig. 1) but having a surface four times larger, the number of each of the three interstitial cell-types was counted; then the total number of these cells in one testis was approximated by multiplying the number counted with a factor, which is proportional to the average length of the short axis of both testes. In this way numbers of interstitial cells were obtained, which relate to the real total number, and which can be compared with each other.

From Text-fig. 7 it follows that:

1. The total number of the three interstitial cell-types increases enormously (cf. the total areas of the columns) as the head appendages develop.
2. In normal birds about 50 per cent. of this increase in size is due to the increase in number of the lipoid cells (cf. Text-fig. 7a, lightly dotted areas).
3. In gestyl-treated birds this increase is only due to the secretory cells, whereas the head appendages have enlarged considerably as a result of the treatment (cf. Text-fig. 7b, heavily dotted and black areas).
4. In normal as well as in gestyl-treated birds, the secretory cells B (cf. Text-fig. 7, black areas) become more numerous as the head appendages increase in size. This was especially perceptible in young gestyl-treated cockerels (cf. the 2 first pairs of columns in Text-fig. 7b).

Conclusion 1. This fact is in favour of Benoit's opinion that the interstitial cells produce the male hormone, but it does not show which of the two cell-types, the lipoid cell or the secretory cell, is responsible for the hormone-production.

Conclusion 2. The increase in number of the lipoid cells might possibly point to the fact that the hormone is produced by the lipoid cells, but the same might be stated for the secretory cells.

Conclusion 3. From this observation it is obvious that the lipoid cells are certainly not directly necessary for the production of the hormone. The obser-vation of Stieve (1923, 1926) who found that in fattened cocks and ganders the number of well-developed cells of Leydig (i.e. our lipoid cells) increases enormously whereas the head appendages do not increase in size, is also in favour of this opinion. Furthermore, one can deduce from Text-fig. 7a that the number of lipoid cells increases as the testis tubules develop. This sug-gests that the testis-tubules do not absorb substances that have been stored
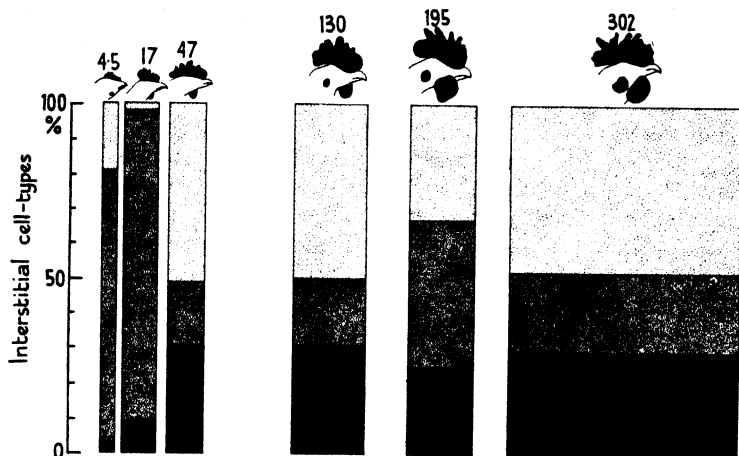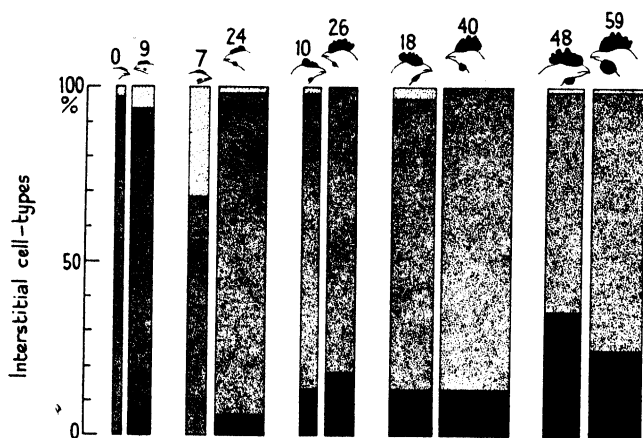
TEXT-FIG. 7. Diagrams showing relative numbers of lipoid cells (lightly dotted), secretory cells A (heavily dotted), and secretory cells B (black) in normal cockerels of 2–200 days (*a*) and in gestyl-treated cockerels. In *b*, the right column of each pair of columns refers to a gestyl-treated cockerel of 14–52 days; the left column refers to a normal control bird. The total numbers of interstitial cells and of each cell-type are shown respectively by the surface area of each column and of its components. The numbers above each head show the relative surface area of the head appendages.

in these lipoid cells. Therefore the quantity of lipoid cells in the testis interstitium seems to depend only on food conditions of the animal, i.e. on the quantity of lipoid that the body can spare for storage at a certain moment.

Perhaps the lipoid cells are indirectly connected with the formation of the hormone, as they might act as storage-cells of special elementary substances. That they contain cholesterol-derivatives (cf. p. 141) points in this direction.

Conclusion 4. The fact that the secretory cells B, representing the most advanced functional stage of secretory cells, increase in number as the head appendages develop, in normal as well as in gestyl-treated animals, is especially important in proving that the secretory cells produce the male sex hormone. The formation of one large vacuole in each cell is in agreement with this. Moreover, the number of secretory cells B is a standard for the quantity of hormone produced.

According to Benoit (1927) the presence of only 0·3 gm. of testis-tissue is sufficient for the complete development of the cock's comb. However, the testes of the cockerels represented by the last column of Text-fig. 7*a* weigh much more. Therefore the large quantity of secretory cells in the testes of these birds points to the following possibilities:

1. In older cockerels there is a large overproduction of the male hormone, this being, however, improbable;
2. Each of the secretory cells B secretes much less hormone in older than in younger birds. This supposition agrees with the fact (cf. p. 143) that, as the cockerels grow older, i.e. from the age of 2 months onwards, lipoid granules originate in these cells. In our oldest cockerels this was the case in most of the secretory cells B. Therefore these cells have the appearance of storage cells (cf. Text-fig. 4 and 6b_1, 6d_1) and only one large conspicuous vacuole reminds one of their original glandular function.

6. DISCUSSION

Finally we will try to give a better insight into the function of the interstitial tissue in cockerels than has hitherto been possible.

It cannot be denied that the storage of lipoids is one of the functions of the interstitial tissue.

The investigations of Benoit have, above all, shown that the endocrine function of the cock's testis must be localized in the interstitial tissue and that glandular cells are found in it. Moreover, the occurrence of the so-called secretory cells B with their large vacuole is in favour of this opinion.

Benoit has also demonstrated a parallelism between the glandular activity of the interstitial tissue and the quantity of male hormone produced. He drew this conclusion from the fact that the total quantity of the interstitial cells increases proportionally to the enlargement of the head appendages, but he does not distinguish the glandular cells with many mitochondria and little lipoid from the lipoid cells, both being considered as secretory cells.

On the contrary, our counts of the interstitial cell-types have shown that Benoit's view, though perhaps not wholly at variance with reality, is much too schematic: there is no reason to call cells, which are packed full of lipoid globules, glandular cells; true connective tissue cells may show the same

phenomenon. In case of the interstitial cells it is therefore likely that these lipoids have only been stored and have not been produced by these cells.

Even if this should be the case, it is not clear what relation exists between the extensive lipoid storage and the formation of the sex hormone. For, in our gestyl-treated cockerels, which had a marked hormone production, lipoid vacuoles in the interstitial cells were practically lacking.

Stieve's fattening-experiments are also in favour of our opinion that the number of the typical lipoid cells relates to the quantity of lipoids which the organism can spare for storage. Therefore we are of opinion that only the secretory cells A and B are responsible for the endocrine function of the testis, an opinion especially founded on the observation that after gestyl-injection the number of these cells alone runs parallel to the enlargement of the head appendages.

Summarizing, we conclude that in embryos and newly hatched male chickens the interstitial testis-tissue has only a trophic function, which is amongst other things evident from the lipoids stored in special lipoid cells, in connective tissue cells and also intercellularly. Then, as the development of the head appendages becomes visible, glandular cells appear which produce the male sex hormone. The storage of intercellular lipoid granules still occurs and lipoid cells are still present at this age. We can neither confirm nor deny Benoit's opinion (1929) that the glandular cells develop from lipoid cells.

During further development, i.e. in cockerels of 1–2 months old, the glandular cells increase in number and become more active, which is shown, e.g., by the formation of a large vacuole in the secretory cells B. In cockerels older than 2 months many of these cells undergo a regression and pass over into lipoid cells.

Finally the situation in the adult cock is reached: only a small percentage of the original secretory cells still function as such, and the others have become storage cells. In old cockerels and adult cocks the accumulation of lipoids is mainly intracellular.

Our opinion confirms that of Benoit (1927a), according to whom a 'secretion de luxe' of testis hormone never takes place. However, we cannot agree with Benoit when he accepts a 'parenchyme de luxe' in cockerels with a testis-weight of more than 0·3 gm. For the tissue, which this author claims to be superfluous, really consists partly of cells which have had a secretory function, but which now possess another function, i.e. the storage of lipoids.

7. SUMMARY

1. The relative volumes of the testes and their components of 31 cockerels, 2–200 days old, were calculated and compared with the size of their increasing head appendages (Text-figs. 1a–d, 2); in addition, the effect of gestyl-administration on testes of cockerels of this age was investigated.

2. Several types of interstitial testis-cells could be distinguished morphologically and physiologically (Text-figs. 3–6 and Pl. 1); these cell-types were studied with different techniques and counted separately.

3. The main types of the interstitial cells are:

(*a*) Lipoid cells, totally packed with lipoid globules. These cells, which are considered by many authors as fully developed Leydig cells, are not directly connected with the production of the male sex hormone; perhaps they have a secondary function in this respect, as cholesterol-derivatives are stored in these cells (Pl. 1, Text-fig. 3*a*).

(*b*) Secretory cells, characterized by the absence of lipoid vacuoles and the presence of numerous granular and filamentous mitochondria. These secretory cells, which produce the male sex hormone, can be divided into secretory cells A (Text-fig. 6*a*) without, and secretory cells B with, one large vacuole (Text-figs. 6*b*, 6*c*, 6*d*).

4. A considerable and partly intercellular storage of lipoids may take place at any age in the intertubular connective tissue (Text-figs. 3–4 and Pl. 1).

5. The number of the lipoid cells depends on the nutritive conditions of the animal and the development of its testes (Text-fig. 7).

6. In older cockerels most of the glandular cells lose their secretory function and pass over into lipoid storing cells.

7. Therefore we agree with Benoit, when he denies the occurrence of a 'secretion de luxe', but we cannot accept the presence of a 'parenchyme de luxe' in the testes of older cockerels.

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DESCRIPTION OF PLATE

PLATE 1. Intertubular testis tissue of cockerels, about 50 days old. *a* and a_2, after Schultz's cholesterol-test; a_1 and c_1, after Sudan III-staining; *b* and *c*, after Champy-fixation and Altmann-staining. *a* and $a_1 \times 100$; $a_2 \times 1,500$; *b*, *c*, and $c_1 \times 1,000$. Head reduced to about ½.

a

a_1

a_2

b

c

c_1

J. W. Sluiter and G. J. van Oordt

PLATE I

Demonstration of Lipine in the Golgi Apparatus in Gut Cells of *Glossiphonia*

BY

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With two Text-figures

INTRODUCTION

THE Golgi apparatus has long been thought to consist in part at least of lipoids. Baker (1944) has summarized and discussed the evidence. The fact that it can be coloured by lipoid colorants (sudan black and sudan III) is a proof that a lipoid or lipoids are present in it. Baker has given very strong evidence, not amounting to histochemical proof, that lecithin or cephalin (or both) are present. The opinions of other workers on the nature of the lipoid contained in the Golgi apparatus are not based on reliable tests.

The purpose of this paper is to present histochemical proof that the Golgi apparatus in the epithelial cells of the alimentary canal of the leech, *Glossiphonia complanata*, contains lipine.

In this paper, the word 'lipoid' is used to include fats and all other substances that occur in plants and animals and resemble fats in solubility. The word 'lipine' refers to lipoids that yield fatty acids, phosphoric acid *or* galactose, and a basic nitrogen compound.

MATERIAL

This study was made on the gut epithelium of the Rhynchobdellid leech *Glossiphonia complanata* (L.). This animal was originally chosen for the suitability of its fat-cells for work on lipines, and because it is common and occurs throughout the year in the adult stage. The Golgi apparatus is visible in the gut after formaldehyde-calcium fixation in unstained preparations mounted in Farrants's medium, and is very easy to demonstrate by a variety of methods. It appears to have the same structure in both stomach and intestine, but although it is easily shown in both by sudan black, the standard silver and osmium techniques give good preparations far more often with the intestine than with the stomach. On the other hand, the acid haematein test rarely gives good results with the intestine, partly because of interference by a diffuse lipine in the cytoplasm.

METHODS

For definitive demonstration of the Golgi apparatus, the following standard techniques were used:

Silver: Da Fano   Osmium: Mann-Kopsch-
        Ramon y Cajal   Weigl (Ludford)
        Aoyama

These were supplemented by Kull's method, after Helly fixation, for mito-
chondria.

For investigating composition, Baker's acid haematein test for lipines
(Baker, 1946) was used, and the following method for sudan black:

(1) Fix, postchrome, wash, embed in gelatine, and cut frozen sections
    exactly as for the acid haematein test.
(2) Leave for a few minutes in 50 and 70 per cent. alcohol.
(3) Transfer to a saturated solution of sudan black in 70 per cent. alcohol
    for 7 minutes or longer (the exact time does not matter; see Lison,
    1936, Baker, 1944).
(4) Pass through three lots of 50 per cent. alcohol, 30 seconds in each.
(5) Rinse in distilled water and mount in Farrants's medium, or counter-
    stain first if desired. One counterstained preparation is useful if the
    tissue is not familiar.

This method is a modification of Baker's. It was used in preference to
Lison's because Dr. Baker informed me that the use of potassium dichromate
helps to make the Golgi apparatus colourable with sudan black in certain
cases in which it is not shown by Lison's technique.

It was found that no difference could be seen between preparations made
in this way and those fixed in formal-calcium with no postchroming, so the
same block was used for acid haematein and sudan black.

The method for nile blue given by Lison (1936) was used, except that
again, as there was no difference between postchromed and not-postchromed
sections, postchromed ones were used. Also, a variety of tissues (sections of
Glossiphonia complanata, *Lumbricus castaneus*, *Dendrocoelum arboreum*, and
liver, lung, and kidney of mouse) were stained in a saturated aqueous solution
boiled with ½ per cent. sulphuric acid (as advised by J. L. Smith, 1908, and
recommended by Lison, 1935*b* and 1936), and compared with others stained
in the untreated solution. Again, no difference could be seen, so the untreated
solution was used, as it appears to keep indefinitely. In addition, the following
method was used.

(1) Prepare a section as for acid haematein.
(2) Stain in saturated aqueous solution of nile blue, 10 minutes.
(3) Leave in 1 per cent. acetic acid for about 18 hours, until the Golgi
    apparatus is pale pink and the cytoplasm colourless.
(4) Wash in distilled water, and mount in Farrants's medium.

Lison (1935, *a* and *b*) concludes, after a very detailed study of nile blue, that
the red coloration is due to nile red, an oxazone occurring as an impurity
in nile blue, and that this behaves like any other lipoid-colouring agent,

e.g. sudan black. It cannot distinguish between the various classes of lipoid. (This conclusion, as Kay and Whitehead (1937) point out, supersedes that given in his book (1936).) He states also that the blue dye is simply a basic dye, and no histochemical conclusion whatever can be drawn from its uses, and (1935a) that it has weak powers of metachromatic staining with ordinary chromotropic elements, the metachromatic colour being *violet bleuâtre*. The method with prolonged differentiation given above does enable one to distinguish between the metachromatic and allochromatic colorations in some cases. It is believed, from some unpublished work, that Lison's conclusions
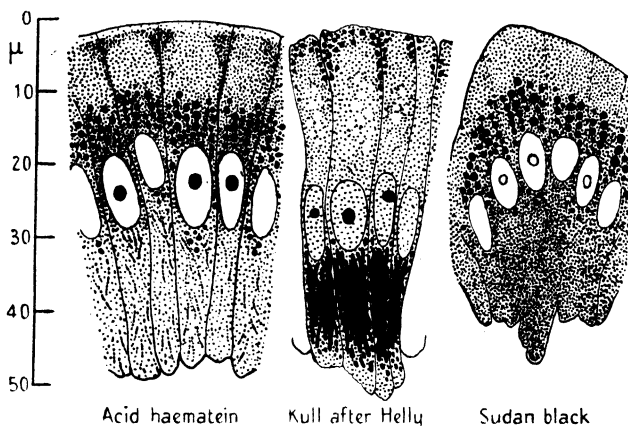
TEXT-FIG. 1. Parts of transverse sections of the stomach of *Glossiphonia*.

are a little over-pessimistic, and that under certain well-defined conditions distinctions can be drawn between certain classes of lipoid; this will be the subject of a separate paper. In the meanwhile, it must be emphasized that no more specificity is claimed for this method than that a red coloration with an aqueous solution of nile blue does show a lipoid. Its value here is purely morphological, as an adjunct to the sudan-black method.

These methods are further discussed below.

RESULTS

(1) *Description of the Golgi apparatus in the alimentary canal of* Glossiphonia

The stomach and intestine of *Glossiphonia* are both very distensible, and the shape of the epithelial cells varies greatly, so that the differences figured by Brumpt (1900) are not always as clear as one might wish. The surest distinction is the position of the lateral diverticula relative to the testes. Those of the stomach lie between or outside the testes, those of the intestine above them.

When the alimentary canal is not distended, both epithelia may be described as columnar (Text-figs. 1 and 2). The nucleus, with a conspicuous

plasmosome, lies in the middle region of the cell. The mitochondria are massed at the base of the cell (most proximal part) and may occupy almost the whole of that region up to the nucleus, but a few are scattered in the distal area, chiefly away from the Golgi region, and close to the sides of the cell. The main mass of the Golgi apparatus, in both epithelia, lies immediately distal to the nucleus (that is, on the side towards the lumen of the gut), but parts may extend down its sides, or even surround it.

The constituents of the Golgi apparatus, as shown in undistended epithelia by the standard methods, appear as numerous uncoloured globules, nearly

Acid haematein   Ramon-y-Cajal
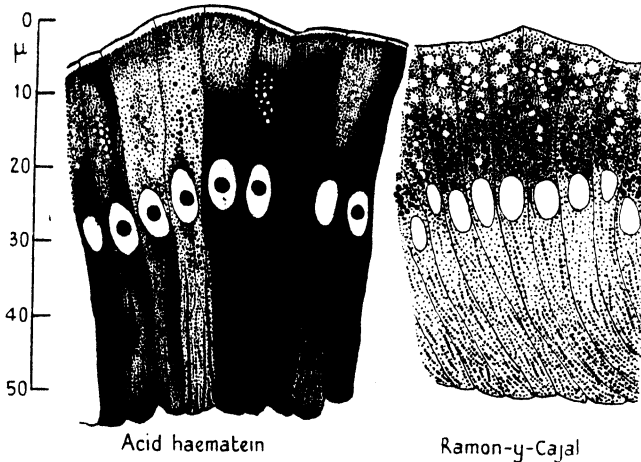
TEXT-FIG. 2. Parts of transverse sections of the intestine of *Glossiphonia*.

every one of them surrounded, at least in part and usually completely, by a shell of strongly osmiophil and argentophil substance. Almost all are arranged in lines parallel with the long sides of the cell. If the globules in one cell are more or less uniform in size, then that size is at the lower end of the range of variation seen. If they are not uniform, then usually a wide range of size is seen, the largest having no shell and being in the most distal part of the cell. The rest of the cytoplasm in osmium preparations is a general grey, usually rather darker in the region of the mitochondrial mass. In toned silver preparations this area is definitely darker than the rest of the cytoplasm. There is no definite evidence for a diffuse osmiophil or argentophil substance specially concentrated in the Golgi region.

The standard techniques usually fail to show anything in the stomach epithelium, but are quite reliable for the intestine. A good Da Fano stomach preparation was obtained, but the Mann-Kopsch technique usually failed completely; and in the only good preparation obtained, the stomach was in the distended state, and the cells so reduced in height that interpretation was very difficult.

(II) *Composition of the parts of the Golgi apparatus*

In both epithelia, after coloration with sudan black, the Golgi apparatus is seen as a crowd of black, usually nearly spherical bodies, arranged in rows as before; occasionally some are apparently coalescing. There is no special indication of a diffuse lipoid between them. A colourless internum is not to be seen.

After acid haematein, it is seen that in the stomach the Golgi apparatus is stained blue quite deeply, the mitochondrial mass much less so. The cytoplasm is greyish from the Golgi area to the free edge, colourless or faintly yellowish elsewhere. The nucleus is almost colourless, except for the plasmosome, which varies from brown to black. The pyridine extraction test gives a completely negative result (except for the nucleus and plasmosome) in both stomach and intestine. As before, the apparatus takes the form of spherules or nearly spherical bodies arranged in rows, more or less, and again, it is difficult to see in any a definite internum. In the intestine, an altogether different appearance is seen. Staining with acid haematein is by far the best method of distinguishing between these two parts of the alimentary canal if there is any doubt. Almost every intestinal cell is full of the blue stain, sometimes so much so that it seems black rather than blue. The plasmosome is again stained, more usually black than brown, and the rest of the nucleus is the only transparent part of the cell. It is usually pale-yellow or yellowish-brown. Nearly always, the whole of the intestine and its diverticula that can be seen in the section is stained like this (and, on a few occasions, the most posterior part of the posterior pair of stomachic diverticula may appear the same), but sometimes a cell here and there, or a small group of cells, is seen to be comparatively clear. In that case the mitochondrial mass is still very darkly stained, and there is a blue patch just inside the free border; but the Golgi region is almost unstained. Often spherules can be seen, but by refraction; a few grains are usually stained, but are very small, and extremely like the small mitochondria in the most distal region. Sometimes, when the whole cell is stained blue, small clear bodies can be seen clustered in the Golgi region. They resemble, quite closely, those seen in that region in unstained sections.

Staining with nile blue by Lison's method shows the Golgi apparatus in both stomach and intestine as spherules (or nearly spherical bodies), darker blue than the surrounding cytoplasm and slightly redder in tone. (As globules of fat in the cell, if present, are coloured red, so that confusion is very unlikely, this is a good, simple method for demonstrating the Golgi apparatus.) Their disposition in the cell is as already described. After prolonged differentiation, quite another picture is seen, but unfortunately a rather faint one. The cytoplasm is now colourless or slightly yellow, the nucleus a very pale blue. The spherules of the Golgi apparatus are seen as colourless or faintly pink globules with definitely pink shells. The internum can now be distinguished clearly from the externum, and it appears to be the latter which is coloured. Under the low powers only, a general diffuse

pinkness is seen in the Golgi region; this is probably due to the colour in the shells of spherules that are out of focus.

<div align="center">DISCUSSION</div>

Lison (1936), speaking of the use of the lipoid colorants, says: 'La spécificité de ces méthodes est parfaite; seuls les lipides donnent des réactions positives.' Nevertheless, if a piece of paper be coloured with sudan black by either Baker's or Lison's methods, it will be seen to retain a faint coloration. And the same depth of colouring will be produced on paper that has first been soaked in pyridine for 24 hours at 60° C.—a procedure that, after suitable fixation, in Baker's pyridine extraction control test results in removing all lipoid except the faintest trace of lipine in the fat cells from a piece of *Glossiphonia*, as may easily be seen by taking a section prepared by the pyridine extraction test method and colouring it with sudan black, instead of staining with acid haematein in the regular way. With such a section, sudan IV, used in the same way as sudan black, gives a quite definite general pink tinge, as it does with paper. It seems reasonable to conclude that if a tissue is not coloured by the very powerful sudan black, a coloration by sudan IV does not show a lipoid. The general pale-pink tinge given by sudan IV has no significance. It is possible, I suppose, that such a powerful colorant as sudan black may actually be colouring an absorbed lipoid, not removable by pyridine, on the paper. However, for very faint colouring by sudan black there remains a slight doubt, unless it disappears after pyridine extraction. As the gut cells of *Glossiphonia* are completely uncolourable by sudan black after pyridine extraction, the point does not arise in this case.

The coloration of paper by sudan black was first noticed by Dr. J. F. A. McManus, who pointed it out to me in conversation.

Nile blue preparations made by the method quoted by Lison show the Golgi apparatus quite clearly, in a darker and slightly redder blue than the surrounding cytoplasm. Tarao (1939) noticed that it was 'blue with red tone' in the liver cell of the mouse. The result with prolonged differentiation makes it clear that this is principally, if not entirely, a case of allochromasy, not metachromasy; but this method is chiefly valuable for showing that the lipoid substance is greatly concentrated in, or confined to, the shell or externum. This makes it very probable that the only reason why the internum was not visible in the sudan black preparations was that the shell was so strongly coloured as to be opaque. That at least the principal lipoid in the Golgi apparatus of the stomach cells is lipine is shown by the positive result with Baker's test. It is suggested, therefore, that the Golgi apparatus in the stomach is composed of rows of (comparatively) fat-free globules with lipoid shells containing lipine.

In the intestine, as exactly the same picture is obtained with sudan black and the standard techniques, the structure appears to be the same as in the stomach, but with this important difference, that where the Golgi apparatus is visible after acid haematein, it is seen to contain little or no lipine as shown by

that test. That it contains much the same amount of lipoid as the stomach Golgi is seen from the sudan black preparations; and as the largest globules, nearest the free border, are not osmiophil or argentophil, it seems likely again that the opacity of the spherules with sudan black is due to intensity of coloration, as in the stomach. It is worth noting that the amount of lipine in the rest of the cell seems to vary inversely with that in the Golgi, there being very little in the stomach cells, and usually a great deal in the intestine. Nevertheless, the bulk of mitochondrial substance appears to be much the same in both. As lipine appears to be particularly plentiful in the actively metabolizing nephridial cells (where it is confined to the very long and numerous mitochondria), and in the fat-cells, where it occurs throughout the cytoplasm, it might be suggested that the abundance of lipine in the intestinal cells is correlated with the active role of the intestine as the food-absorbing region (see Brumpt, 1900).

SUMMARY

(1) The standard silver and osmium techniques show a typical Golgi apparatus in the epithelial cells of the stomach and intestine of the leech, *Glossiphonia complanata*.

(2) Histochemical studies reveal the presence of lipoids in the Golgi region of these cells. In the stomach, part, at least, is lipine. (In the intestinal cells, the rest of the cytoplasm contains much lipine, but the Golgi apparatus little or none that can be shown by the method used.)

(3) The Golgi apparatus in these cells appears to consist of rows of non-lipoidal spheres, each with a lipoid coat. In the stomach, this coat contains lipine.

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Note on the Cytological Localization of Alkaline Phosphatase

BY

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IN comparing various techniques for the microchemical demonstration of alkaline phosphatase it was observed that with the method of Menten, Junge, and Green (1944) nuclear phosphatase cannot be demonstrated in epithelial cells, whereas with the Gomori–Takamatsu (1939) technique both nuclear and cytoplasmic elements (e.g. brush borders and the Golgi zone) of suitable organs show the presence of the enzyme. The substrate in the method of Menten, Junge, and Green is Ca α- or β-naphthylphosphate in the presence of α-naphthyl-diazonium hydroxide. In the Gomori technique the substrate is sodium-β-glycerophosphate. The failure of nuclear phosphatase to be demonstrated by the first method might be caused by:

I. Failure of nuclear phosphatase to act on Ca α-naphthylphosphate.

II. Inactivity of nuclear phosphatase at the low temperature ($10°$) at which the diazo mixture has to be kept.

III. Irreversible inactivation by one of the constituents of the diazo mixture or

IV. Inhibition by their presence during incubation.

V. Failure of the diazo method to detect the relatively low concentrations of phosphatase in the nuclei.

To ascertain which of the above postulates is correct the following experiments were performed: Paraffin sections of guinea-pig small intestine and rat kidney fixed in 80 per cent. alcohol were placed in the substrate of Menten, Junge, and Green at $10°$ C. for 4 hours.

This mixture tends to lose its activity, by-products being precipitated, and was therefore renewed after 2 hours. Other slides were incubated in Gomori's substrate at the same temperature. All media were at pH $8·5$ and contained $0·2$ per cent. $MgCl_2$.

The sections incubated in the diazo mixture showed a heavy deposit of dye in the free border of the intestinal epithelium and of the convoluted tubules of the kidney cortex, the nuclei being negative. The Gomori slides showed blackening of the nucleoli and the nuclear membrane as well as of cytoplasmic elements. Thus nuclear phosphatase does act on glycerophosphate at $10°$ C., and postulate II does not apply.

One of the slides was now transferred from the diazo to the Gomori sub-strate at the same temperature and incubated for 4 hours. The same degree of blackening of the nuclei was produced as in the sections not given a prior incubation in Menten, Junge, and Green's medium, showing that the activity of nuclear phosphatase is unimpaired by previous contact with the diazo mixture (III).

To investigate cause IV experiments were done to see whether α-naphthyl-diazonium hydroxide (*a*) or Ca α-naphthylphosphate (*b*) inhibit the hydrolysis of glycerophosphate (*c*) and whether glycerophosphate inhibits the hydrolysis of Ca α-naphthylphosphate. It was found that (*a*) inhibits hydrolysis of (*c*) and (*c*) inhibits hydrolysis of (*b*) as indicated by the lack of formation of precipi-tates in the sections. It must be noted that cytoplasmic as well as nuclear elements failed to blacken. But this lack of precipitation does not necessarily signify, in this instance, failure to hydrolyse: it may only indicate that the products of hydrolysis are not precipitated under these conditions.

It has been shown that phosphatase in the striated border of kidney tubules is more easily destroyed by heat than nuclear phosphatase (Danielli and Catche-side, 1945). There is thus considerable evidence that nuclear and extranuclear phosphatase behave as different enzymes in the given epithelia. Moreover, Dempsey and Deane (1946) have demonstrated cytochemically that a number of phosphatases acting on different substrates coexist in the duodenal epithelium.

The above results are in contradiction to Menten, Junge, and Green's (1944) statement that the distribution of alkaline phosphatase in the kidney appears to be the same whether their method or that of Gomori be used.

However, the fact that polymorph nuclei in glomerular capillaries of guinea-pig kidney sections show deposits of azo-dye is in favour of postulate V, viz. that there might be insufficient nuclear phosphatase in the epithelial cells examined. Danielli (1946) in a critical study of histochemical methods relating to phosphatase suggests that the diazo technique might be inferior to the Gomori method in revealing sites of low phosphatase activity. To investigate this further, sections known to be very rich in nuclear phosphatase (as judged by a heavy deposit after short periods of incubation with Gomori's substrate) were treated by the diazo method. The following tissues were chosen: epiphyses of long bones of growing mice and the costo-chondral junctions of rat ribs (decalcified by my method [Lorch 1946]), calcifying cartilage of dogfish embryos, and kidneys of various fish known to contain much nuclear phosphatase in the interstitial cells. In all tissues examined some of the nuclei were positive.

SUMMARY

It is concluded that there is no definite proof that nuclear phosphatase as such is qualitatively different from extra-nuclear phosphatase, but that the method of Menten, Junge, and Green is not capable of revealing sites of low concentration of phosphatase in which respect it is markedly inferior to the Gomori–Takamatsu technique.

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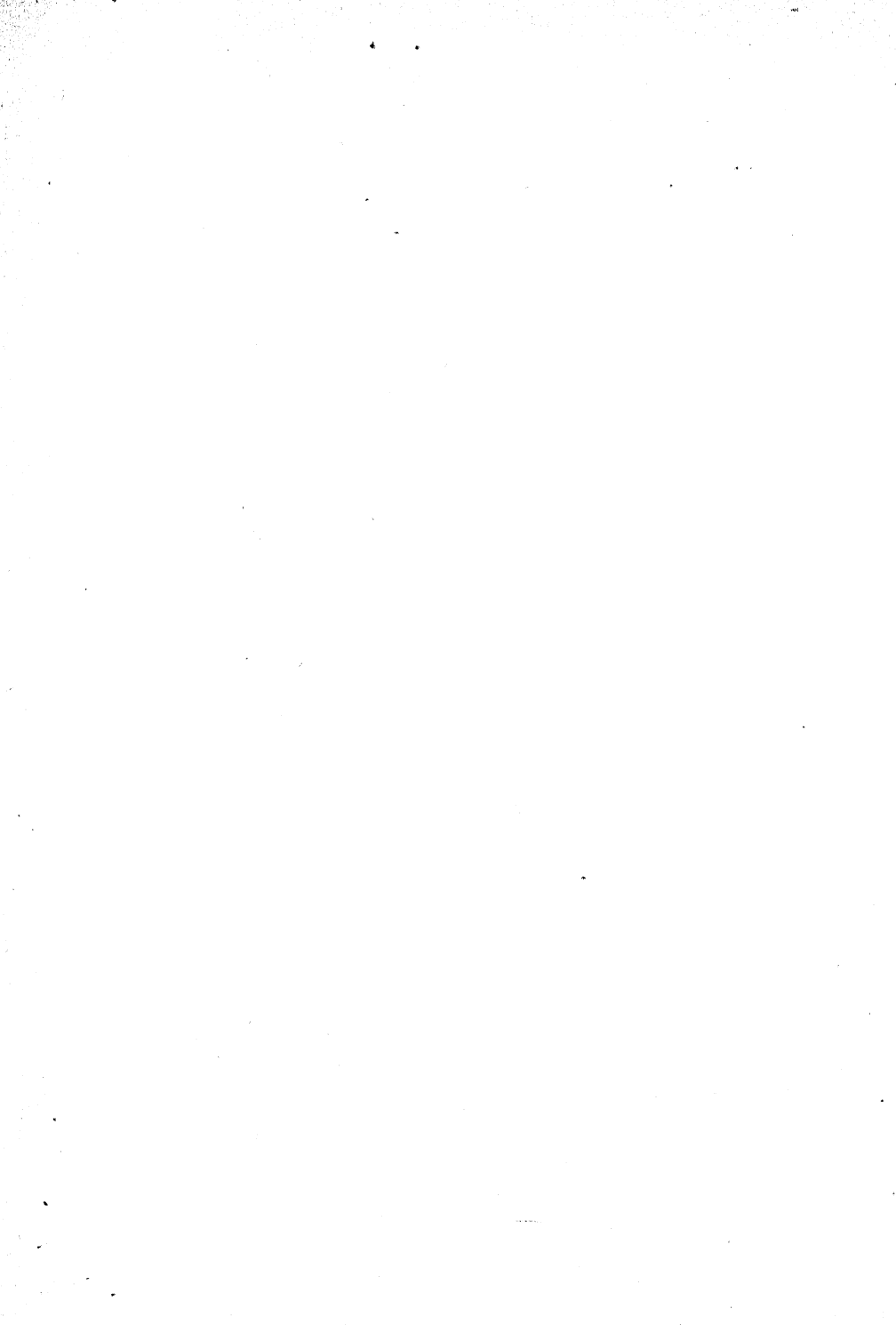
The Use of Cajuput Oil in Microscopy

BY

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DURING the world war 1914–18 our stock of xylene (xylol) in Java ran low; moreover, the small quantity that was left changed into a thick syrupy fluid which was useless as a medium for mounting water-fixed preparations in balsam. Chemists told us that the xylene had polymerized. In text-books of microscopical technique cedar-wood oil is recommended as a substitute for xylene in some cases. However, this oil was still scarcer in Java than xylene; therefore we resolved to try another ethereal oil, namely, cajuput oil. Of this oil, a product of the Malay Archipelago, unlimited quantities were available. It soon proved to be not only a good substitute for xylene, but in some cases superior to it.

Cajuput or cajaputih oil is obtained by distillation from the leaves of *Melaleuca leucadendron* L. (*Melaleuca cajuputi* Roxb.). 'Cajaputih' is apparently a misnomer for the Malay *kaju putih*, i.e. white wood. The oil is manufactured in the island of Buru (Moluccas) and, to a less extent, in the western part of the neighbouring island of Ceram. The oil on the market is green, owing to a small admixture of copper salts derived from the distillation apparatus. Oil containing no copper is pale brown. In the Far East, particularly in the Malay Archipelago, it is a very popular medicine for almost any ailment. As a rule it is applied externally for massage, but a few drops may be swallowed in cases of abdominal complaints. Being accustomed to it, the patients ask for the green oil; the brown is not popular, and so only the green oil containing copper can be had on the market. According to Lee and Mayer (1898) and to Ehrlich (1910) cajuput oil is recommended by Carnoy and Lebrun and by Nissl as an intermedium before imbedding in colophonium.

A real advantage of cajuput oil over xylene is its ability to absorb small quantities of water. If films or sections are passed from alcohol to balsam via xylene, the alcohol must be nearly free of water; otherwise water is deposited in the preparation and renders it cloudy. Now in the moist and warm climate of Java it is very difficult to keep alcohol water-free, even with burnt copper sulphate on the bottom of the container. On the other hand, slides may be transferred from alcohol, containing a small amount of water, to cajuput oil and afterwards to balsam without getting turbid.

The capacity of cajuput oil to absorb small quantities of water proved to be useful especially in mounting wet-fixed, Giemsa- (or Leishman-) stained preparations. It is usually recommended to pass such slides successively

through: (1) acetone 95 p., xylene 5 p.; (2) acetone 70 p., xylene 30 p.; (3) acetone 30 p., xylene 70 p.; and (4) pure xylene. Slides ought to remain for a very short time only in the fluids containing acetone as otherwise the preparations are decolorized. Sometimes too much stain is extracted before complete dehydration. If cajuput oil is used the slides may be passed into the oil after a very short stay in acetone (say, one second); after which they may remain many minutes in the oil for complete dehydration without becoming decolorized. The degree of decolorization of overstained films can be watched easily by mounting them in cajuput oil under a cover-glass; if insufficiently decolorized they may be passed at once for one or more seconds to acetone.

Another advantage of cajuput oil over xylene I experienced when mounting chitinous structures, mainly hypopygia of Culicids, in balsam. Not only is turbidity by deposit of water avoided, but also the oil does not render the chitin brittle. This is important, because the position and the arrangement of the parts of the hypopygium will often have to be corrected with a dissecting needle after it has been transferred to a drop of balsam. Xylene-treated chitin is very brittle; even a slight touch may damage it. This will not happen if xylene is substituted by cajuput oil. For the same reason the oil may serve as an intermedium between alcohol and paraffin in imbedding chitinous structures in the latter. Moreover, in this case it has the advantage of being more volatile than, e.g., cedar-wood oil. Therefore it is more easily eliminated from the paraffin by heating. Paraffin sections still containing a trace of cedar-wood oil will expand greatly when flattened on water.

It is quite possible that in the above-mentioned cases other ethereal oils are as good as cajuput oil. At any rate the latter is one of the least, if not the least, expensive. Before the last war the price of 1 litre (1·76 pints) in Amsterdam was fl. 1·70 (i.e. about 3s. 6d.).

SUMMARY

Cajuput oil has some advantages over xylene for mounting preparations in balsam. Cajuput oil can hold some water without getting turbid; it does not render chitinous structures brittle; and it can be used with advantage in the mounting of wet-fixed, Giemsa-stained slides.

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The Development and Affinities of the Pauropoda, based on a Study of *Pauropus silvaticus*

BY

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PART I

With 9 Plates and 22 Text-figures

CONTENTS

INTRODUCTORY

IN the Pauropoda we have a group of very small and obscure myriapods in whose morphology, there is reason to suspect, some unusually primitive features are revealed. Their development may therefore be expected to throw light on some major problems connected with the evolution of the Myriapoda and their derivatives, the insects, and it is mainly with this end in view that the present work has been undertaken.

The gross anatomy of the adult animal is now fairly well known, having, since Lubbock's original work of 1868, been investigated by Schmidt (1895), Kenyon (1895), and Silvestri (1902). But there are many features of their structure, notably the all-important character of the mouth-appendages, upon which we are quite inadequately informed, but on which the assessment of affinities in large measure depends. In the following account, therefore, I have often found it necessary to prefix the embryological description of various organs with a brief, or at times even lengthy, statement on their adult morphology.

Accounts hitherto given of the development of *Pauropus* consist almost exclusively of a description of the external characters of various larval phases. The anamorphosis was discovered by Lubbock. Later writers—Latzel, Silvestri, Harrison, and others—have described more fully the individual larvae, though without adding much of significance to Lubbock's account. The eggs were discovered by Ryder as long ago as 1879. Harrison (1914) collected a large series of eggs of the Australian *Pauropus amicus*, but recorded nothing of their development beyond the fact that they appeared to undergo total cleavage. He made, however, the important observation that the young *Pauropus* left the egg, not as a larva, but as a motionless embryo, enclosed in an embryonic cuticle 'covered with long, tapering, cylindrical hair-like outgrowths'; and it is clear from his account that he had before him the 'pupoid'

phase. Beyond this, no observations on the development of *Pauropus* seem to have been recorded.

BIONOMICS AND LIFE-HISTORY

Pauropus silvaticus inhabits the heavily timbered mountainous rain-forest country of Victoria. A taxonomic description of this species has been given in an earlier paper (Tiegs, 1943). It is a small, agile creature, and even when fully grown rarely exceeds 1·1 mm. in length. The animals may be found by turning over rotting logs, or by scraping away the leaves on the forest floor. But they also enter rotting timber, choosing by preference decaying trunks of tree-ferns (*Alsophila*, *Dicksonia*), and here they are sometimes present in great abundance. They favour damp localities, avoiding both dry and excessively wet places.

The associated microfauna comprises various species of Symphyla, Collembola, pseudoscorpions, mites, and small insect larvae; but from all these they are readily distinguished, even to the unaided eye, by their active mouse-like movements.

They are light-shy creatures, and, if dropped in an exposed place, commonly feign death. From the agility of their movements Latzel (1884) suspected that they were probably predaceous. *Pauropus silvaticus* I have, however, kept and bred in captivity for months in receptacles containing only fragments of decaying tree-fern; nor have I ever seen them, in the field, devouring the remains of other small creatures, though the reverse is sometimes the case, carnivorous mites and pseudoscorpions frequently taking toll of the larvae and even of the adults.

Oviposition takes place in early and middle summer, the eggs being laid singly in secluded clefts in the decaying vegetation in which the animals live. They are minute and spherical, measuring usually about 0·11 mm. in diameter, and under a hand-lens appear a smooth pearly white. In another species (*Pauropus amicus*) Harrison (1914) found that the eggs were laid in clumps, over which the female then mounted guard; *P. silvaticus* does not, however, display this maternal instinct, nor are the eggs ever laid other than singly.

Development within the egg occupies a minimum of 12–13 days; already on the tenth day a wide rent has appeared in the chorion, but it is not till 3 days later that the 'pupoid' phase is entered upon. The 'pupa' is still a quiescent stage, and in this condition the young animal remains for about 3 or 4 days longer. Eventually its cuticle splits along the mid-dorsal surface, and the larva emerges.

The first instar larva is a minute white creature, measuring not more than 0·25 mm. in length at the time of emergence. As Lubbock first observed, it presents already the main characters of the adult animal, but has only three pairs of legs. Moving at first sluggishly, it gains both in size and in strength, and soon acquires the agility of the adult. After about 3 weeks it moults, and discloses the second instar larva.

The latter, at the time of its first appearance, measures about 0·3 mm. in length; it possesses five pairs of legs (there is no larva with four pairs). The second larval stadium has a duration of about 4 weeks.

The third instar larva, which measures, at the time of its first appearance, about 0·5 mm. in length, has six pairs of legs. The duration of this stadium is about four weeks.

Two additional pairs of legs develop during the third larval stadium, the fourth instar larva having therefore eight pairs of legs. It measures 0·7–0·8 mm. in length; the duration of the stadium is about three weeks. At the end of this period the larva again moults, and so the adult stage with nine pairs of legs is attained. I have examined large numbers of exuviae collected from decaying wood in which the animals were abundant, but have failed to find any with more than eight pairs of legs; it would seem, then, that moulting ceases after the full complement of legs has developed.

The longevity of the animals is unknown; I have had some in captivity for nearly a year.

The duration of the larval period, viz. about 14 weeks, is much less than might have been expected from the statement of Lubbock that a first instar larva of *P. huxleyi*, kept in captivity by him for 6 weeks up to its death, failed to moult. My own observations have been made on only a few larvae in captivity. The duration assigned to the successive instars is approximate only; for the larvae are apt to disappear for days into clefts in the rotting timber where it is not possible to follow them without risk of crushing their excessively fragile bodies.

METHODS

Owing to their unfortunate habit of laying their minute eggs singly in hidden clefts in the rotting timber in which they live, the collecting of an adequate supply of eggs is a very laborious task. About 200 eggs were obtained by carefully searching through infected logs brought into the laboratory; but I have also kept many animals in captivity, and from these have obtained a large supply of eggs. From the latter some approximately timed stages have been obtained; when ages are assigned in the text to particular embryos, they refer, in all cases, to laboratory-laid eggs at a temperature of 17°–20° C.

For fixing the eggs and pupae I have used Carl's fluid (acetic acid 2 parts, formalin 6 parts, alcohol 15 parts, water 30 parts). This fixative does not collapse the eggs, as does the more anhydrous Carnoy's fluid; it penetrates rapidly through the egg-membranes, which it is not necessary to puncture, about 15 minutes usually sufficing for fixation, after which the material is stored in 70 per cent. alcohol. Although the fixation is generally very good, it has the defect that it sometimes causes shrinkage of the already minute embryo. For larvae and adults this fixative is inadequate, since they are not wetted by it; Carnoy's fluid, on the other hand, gives excellent fixation, without the distortion that it produces in eggs.

As a stain for whole embryos and larvae I have used Auerbach's methyl green-acid fuchsin mixture. To admit the stain the egg-membranes or chitin of the larva are first punctured with a very finely ground needle. It is not necessary to unsheath the eggs for examination, for, after clearing, the embryo is visible in all detail through the delicate chorion.

Sections have, in all cases, been cut from celloidin-paraffin double-embedded material; for staining the sections I have used iron haematoxylin.

OBSERVATIONS ON EMBRYONIC DEVELOPMENT

1. *The Egg (Structure, Oogenesis, and Fertilization)*

The egg is spherical and minute, measuring rarely more than o·11 mm. in diameter, while at times eggs as small as o·09 mm. are met with. The chorion is soft, and is covered with a multitude of minute spines, amongst which there is a single spine of much greater size (Text-fig. 1). In the great majority of eggs in which an embryo is present, this enlarged spine accurately marks the anterior pole of the egg; yet I have a few eggs in which this relation is quite clearly not observed.

The interior of the egg is occupied by yolk, the separate grains of which are supported within a fine framework of cytoplasm. A periplasm is not present, nor is there a vitelline membrane.

I am able to give only a meagre account of the meiosis, for I have had the usual difficulty of obtaining a sufficiently complete series of very early eggs. Such early stages as I have obtained were mostly got by frequent search in a receptacle into which some 80 adult animals had been placed. They lay, however, only at long intervals, and, moreover, only in the dark.

During the period of yolk-accumulation, and, indeed, up to a little before laying of the egg, the nucleus is in the germinal vesicle condition (fig. 2, Pl. 1). It measures about 25μ in diameter, displays several clumps of deeply chromatic substance, and a rather diffuse, weakly staining coagulum, within which only very doubtful indications of chromosomes are visible.

Shortly before the egg is laid the germinal vesicle gives way to a very characteristic phase, of which I have many examples, and which is shown in figs. 3 and 4, Pl. 1. The chromosomes have reappeared, and indeed with great clearness. They occur as 'tetrads', and of these there are thirteen. The diploid chromosome number is therefore twenty-six. They are not scattered at random, but lie uniformly in one plane. This is well seen in fig. 4, Pl. 1, which shows six of the thirteen bivalent chromosomes. Fig. 3 shows them in polar view. In favourable preparations spindle-fibres are faintly visible (fig. 4, Pl. 1). It is the metaphase of the first meiotic division.

I have no stage between this and the germinal vesicle. Presumably it has arisen by disruption of the latter, and the reappearance of the chromosomes within a central accumulation of cytoplasm; for neither in texture, nor in its further history, is the spherical body within which the chromosomes are lodged suggestive of nuclear material.

In the youngest-laid egg which I have obtained (it is probably not more than a few minutes old) this mass of cytoplasm has moved to the margin of the egg (fig. 5, Pl. 1), and within it the ensuing meiosis takes place. The chromosomes still lie, as tetrads, in one plane, but have begun to shrink a little. A reduction division now ensues, the chromosomes having shrunk still further into almost spherical masses. The anaphase of the reduction division is shown in fig. 6, Pl. 1; the division-plane, it will be observed, is not tangential, as usual, but radial. A polar view of this phase is seen in fig. 7, Pl. 1, and clearly shows the reduction in chromosome number.

Owing to lack of material I am unable to describe the process of second polar body formation. The polar body itself, however, and also the first, are clearly distinguishable up to about the time the male and female pro-nuclei have fused. Fig. 8, Pl. 1 shows them in a section that grazes the surface of the egg, and they are also indicated, in part, in figs. 9, 10, Pl. 1. From fig. 8, Pl. 1 it is evident that neither in the first nor in the second polar body do the chromosomes reassemble into a resting nucleus, and they do not even become enveloped in a nuclear membrane. The cytoplasm of the two is only very incompletely constricted, and, moreover, in neither polar body does it separate from the egg, but remains within its surface layer. The first polar body does not undergo further division.

Degeneration of the polar bodies usually sets in very early. In some eggs, even at the first cleavage, they can no longer be seen; yet in other cases they survive to the time when as many as eleven nuclei have appeared (figs. 40, 41, Pl. 4).

Fig. 9, Pl. 1 shows a section of an egg, of unknown age, in which the fully formed male and female pro-nuclei are for the first time distinguishable. The pro-nuclei lie within a central aggregation of cytoplasm, and within each the chromosomes are visible as delicate threads. The smaller of the two is probably the female nucleus, since it is nearer the polar body. I have not been able to recognize the sperm-head in any egg, and only very doubtful indication of a male pro-nucleus earlier than that just described.

The two pro-nuclei soon fuse to form the zygote nucleus (fig. 10, Pl. 1). The latter is a typical resting nucleus, within which chromosomes are not visible, the chromatin being concentrated partly within a small nucleolus, but otherwise scattered evenly in small grains throughout the nucleus.

2. *Segmentation of the Egg, and Formation of the Gastrula*

The large zygote nucleus now divides into a pair of cleavage-nuclei, which separate from one another, and move into opposite halves of the egg. Between the nuclei the remains of the spindle may persist for a time within the yolk (fig. 38, Pl. 4). In some eggs the cytoplasm which invests these nuclei is abundant, in others it is present in small amount only. From the delicate cytoplasmic reticulum in the interior of the egg a thin partition then forms, dividing the egg into its first two blastomeres, and these are roughly equal in size (fig. 11, Pl. 1). The groove demarcating

the blastomeres externally varies from one that is scarcely noticeable to a pronounced fissure.

I have only a single egg at the succeeding 4-cell stage (fig. 12, Pl. 1). In this particular egg the second cleavage has not been a complete equatorial cleavage, but the first two blastomeres have divided independently in planes at a right angle to one another and to the first cleavage plane. Whether the 4-cell stage is always arrived at in this way is, however, uncertain. A section through this egg is shown in fig. 39, Pl. 4.

I have not been able to recognize any further plan in the cleavage; for it is impossible to determine completely the orientation of the egg, and, moreover, partitions between the blastomeres may themselves be difficult at times to recognize. Cleavage, from now on, is markedly unsynchronized, both in respect to the incidence of nuclear division, and to the formation of cell-partitions. I have, for example, one egg in which, though there are eight nuclei present, only six blastomeres, of unequal size, have become demarcated; in another egg, with eleven nuclei, seven are undergoing division and the other four are resting.

Some eggs in successive stages of cleavage are shown in figs. 13–17, Pl. 1, and figs. 40–3, Pl. 4. Fig. 13, Pl. 1 depicts a 6-blastomere egg. An 8-blastomere egg is shown in fig. 14, Pl. 1, and a section through this egg in fig. 40, Pl. 4; the section shows, in the middle of the egg, the beginning of a segmentation cavity, where the innermost ends of the blastomeres have separated a little from one another. Fig. 41, Pl. 4 represents a section through an egg in which eleven nuclei are now present, of which some, but not all, are in mitosis; it will be noted that the plane of cleavage is radial, the spindle-axes lying tangentially. In this egg the segmentation cavity has not yet appeared. A more advanced egg, with 16 nuclei, but still only 11 blastomeres, is shown in fig. 15, Pl. 1. An egg with about 24 blastomeres is shown in fig. 16, Pl. 1, and a section through this egg in fig. 42, Pl. 4; the blastomeres are now typical 'yolk-pyramids', with the nuclei located in a clump of cytoplasm near the periphery, but still separated from the surface of the egg by several yolk-grains. There is now a conspicuous segmentation cavity. This simple cleavage, with the formation of 'yolk-pyramids' alone, ends at about the 40-5-cell stage. An egg at this phase of development (late blastula) is shown in fig. 17, Pl. 1, and a section through it in fig. 43, Pl. 4. The 'yolk-pyramids' have now grown narrower, and the segmentation cavity is even larger than in the foregoing egg. The nuclei are almost at the surface of the egg, with usually only a single layer of yolk-grains intervening.

By the time the number of cells has doubled, i.e. at about the 80-cell stage, a noteworthy change has taken place in the segmenting egg. An entire egg at this stage is shown in fig. 18, Pl. 1; a section through it in fig. 45, Pl. 4. The former segmentation cavity is now occupied by a clump of yolk, within which one or at most two large pale nuclei can be seen. As its further development shows, the central clump constitutes the endoderm; it is still very deficient in cytoplasm, and is recognizable as a distinct cell only by its large nucleus or

nuclei. The nuclei of the 'yolk-pyramids' have now mostly moved into the surface cytoplasm of the egg, and are flattened and much diminished in size. With the obliteration of the segmentation cavity a peripheral zone of large vacuoles appears in the yolk, and remains, for a time, a very conspicuous feature of the egg.

It is probable that the endoderm has arisen by the tangential, instead of radial, division of one or two 'yolk-pyramids'. I have not been able to observe the actual mitosis. I have, however, one egg in which the endoderm is seen in process of formation (fig. 44, Pl. 4); there are present 75 yolk-pyramids, each with its small nucleus located in the peripheral cytoplasm: the endoderm cell has partially obliterated the segmentation cavity and its outline is only vaguely defined, but its nucleus, already large, still lies towards the periphery of the egg. Whether the second endodermal nucleus arises in a similar way, or by division of the first, I have not been able to determine; the latter is probably the case, for the two nuclei are always found very close together.

There seems to be justification for regarding the stage, which is thus arrived at, as a gastrula (see further, section 5). This gastrula endures until about 160–200 cells have appeared in the outer layer. A drawing of the mature gastrula is shown in fig. 19, Pl. 1, a section through it in fig. 46, Pl. 4. In entire stained embryos the nuclei, further diminished in size, are now seen in great profusion at the surface, where yolk-grains are also still visible. The nuclei appear rather more crowded toward the future ventral pole of the egg. The peripheral cytoplasm within which they lie has begun to increase in concentration and thickness. Each nucleus, although lodged within the outer cytoplasm, is still the nucleus of a 'yolk-pyramid'. The latter cells have now acquired an almost columnar form, and their walls are becoming faint and difficult to distinguish, being quite imperceptible in surface views of whole eggs. In the central endoderm still not more than two large nuclei are present.

3. *Passage from the Gastrula to the Blastoderm Phase*

This takes place during the third day. At the periphery the cytoplasm continues to increase in quantity, and in some eggs, but not in all, attains even a considerable thickness. Within this peripheral cytoplasm the cleavage nuclei are almost all contained; the only exceptions are the nuclei of the central clump of endoderm and, in some eggs, a few enlarged nuclei of the 'dorsal organ' (see section 14 (vii)). Amongst the peripheral nuclei irregularly scattered mitoses are abundant.

The most noteworthy change in the interior of the egg is the breakdown of the partitions which have hitherto separated the 'yolk-pyramids', and therewith the yolk reverts to its originally unsegmented state. The wall which invests the central clump of endoderm can, however, often still be seen. Total cleavage of the egg thus gives way to a cleavage confined to the layer of surface cytoplasm. In good preparations cell-boundaries are easily distinguishable in this peripheral cytoplasm; but it is to be noted that there are no

cell-walls delimiting the latter from the intravitelline reticulum of protoplasm. The blastoderm, which thus develops, soon secretes a cuticle on its surface. This blastodermic cuticle is a very thin and perfectly smooth membrane, without any surface-sculpture. Unlike the chorion, it resists boiling with caustic soda.

A section through an early and still very thin-walled blastoderm is shown in fig. 47, Pl. 4; sagittal sections through two more advanced blastoderms in figs. 48, 49, Pl. 4. The appearance of the entire blastoderm is shown in fig. 20, Pl. 2. The surface aggregation of nuclei is evidently much more marked than in the gastrula, and the yolk has completely disappeared from the surface of the egg. The great change that has taken place in the surface epithelium will be seen by comparing figs. 50 and 51, Pl. 5, these drawings having been made from a mature gastrula and a mature blastoderm respectively, at identical magnification, and representing fragments of sections grazing along the surface of the egg. As the sections in figs. 47, 48, 49, Pl. 4, show, the internal wall of the blastoderm presents a very ragged appearance, for even in advanced blastoderms there are still no cell-walls delimiting it from the internal protoplasmic reticulum.

In actual appearance there is much variation between individual blastoderms. In some the epithelium remains quite thin, in others it becomes surprisingly thick. There is also frequently seen, even in young blastoderms, a precocious distinction into a thinner dorsal and a thicker ventral half; yet other, even mature, blastoderms may be of uniform thickness throughout. Eventually, however, in all cases a ventral thickening develops, in preparation for the impending formation of the germ-band.

In the central clump of endoderm there are still present not more than two nuclei; the cytoplasm has, however, begun to increase in quantity.

4. *Differentiation of the Germ-band from the Blastoderm, and the Formation of the Yolk-cells and Mesoderm*

By the fifth day the ventral thickening and attendant dorsal thinning-out of the blastoderm, referred to in the preceding section, have become much accentuated. Out of the lower thickened half the germ-band will develop; the dorsal thinner half will become the provisional body-wall. In whole preparations the two regions of the blastoderm are easily distinguishable, not only by differences in thickness, but by differences in spacing of nuclei, which are much sparser over the thin-walled half (fig. 21, Pl. 2).

Sections of the egg at this stage of development show the yolk-cells and mesoderm in process of formation. Already in late blastoderms isolated cells have separated from the epithelium, and are in process of invading the yolk (figs. 48, 49, Pl. 4); they soon become distinguishable by their large clear nuclei, with conspicuous nucleoli, and in this respect resemble the future yolk-cells, of which they are the forerunners, and out of which the fat-body will later develop. In blastoderms with ventral thickening these cells have increased in number, and a few may even be encountered deep in the yolk.

These 'yolk-cells' are not delimited by cell-walls, but are part of the intravitel-line protoplasmic reticulum. The cells of the blastoderm, on the other hand, have now become sharply demarcated by cell-walls from this reticulum, only the few cells from which the 'dorsal organ' (see section 14 (vii)) will develop, remaining in continuity with it. The 'yolk-cells' arise at random from any part of the blastoderm; the mesoderm forms only from its ventral thickened half, and it is to this process of mesoderm formation, quite as much as to the local aggregation of blastoderm-cells, that ventral thickening is due.

Mesoderm formation does not, however, take place uniformly over the entire thickened ventral half, but is restricted to an area that corresponds roughly to the limits of the future germ-band; we find therefore, in sections that are taken 'horizontally' across the region of thickening (fig. 52, Pl. 5), that there is, on each side, a strip of intervening blastoderm where there is no mesoderm formation. In external views of whole embryos this zone without mesoderm is not distinguishable.

Where mesoderm formation is in progress, the cell-nuclei are piled several deep in the blastoderm (fig. 52, Pl. 5). Individual cells now separate from the latter, and gradually form into an irregular and broken layer of cells which remain adherent to the outer layer (fig. 53, Pl. 5). These mesoderm cells are readily distinguishable from the yolk-cells by their smaller and darker nuclei.

While the mesoderm is still in process of forming, the thickened epithelium on the lower half of the egg begins to acquire the contour of the germ-band. This is brought about by the lateral encroachment of the provisional body-wall on to the lower half of the egg, by thinning out of the mesoderm-free area; at the same time the zone of thickened epithelium spreads farther up over the anterior and posterior poles towards the mid-dorsal surface. A whole embryo at this stage of development is shown in fig. 22, Pl. 2, a sagittal section along it in fig. 54, Pl. 5. In this embryo stomodaeum formation is in progress. The stomodaeum is a small conical ingrowth of ectoderm, not yet showing a lumen, and therefore not visible in whole preparations. The yolk-cells are now scattered through the yolk. The mesoderm extends along most of the germ-band, and partially encircles the stomodaeum. Behind the latter it is heaped up, and this local accumulation of mesoderm is the source of the future pre-oral mesoderm. Farther to the rear the mesoderm forms a layer of irregu-larly scattered cells, with small cell-aggregations particularly along the lateral margins of the germ-band where the future somites will form.

The description of the mesoderm is continued in section 7.

5. *Gastrulation and the Formation of Germ-layers in Myriapods and Insects*

In the early development of myriapods and insects we are confronted with a type of ontogeny whose meaning has been the subject of much speculation. What is the significance of the blastoderm? Is there a gastrula? Does development proceed in conformity with germ-layer principles? *Pauropus* shares with other myriapods and with insects some of the features

of their specialized development; but the presence of a thinly veiled gastrula is unexpected. Its existence offers new scope for discussing the above questions.

In all species of *Peripatus* whose development has been examined, a gastrula can be recognized. In *P. capensis* it is an epibolic gastrula, in which both archenteron and blastopore are present (Sedgwick, 1885). The two other' yolk-deficient species whose development is known—*P. edwardsii* (Kennel, 1884), *P. imthurni* (Sclater, 1888)—seem to lack a blastopore, and in the former even the appearance of the archenteron is delayed. Special significance attaches to the species with yolk-laden eggs. Of these the early development of two has been examined—*P. novae-zelandiae* (Sheldon, 1887–8), *P. weldoni* (Evans, 1901); in both there is a long ventral blastopore, from which endoderm grows inwards around the yolk, but an archenteron does not form. In all the species the mesoderm is found to arise from the neighbourhood of the blasto-pore. The formation of an embryo then proceeds without the intervention of a blastoderm phase.

In *Pauropus* also, with from 80 to 200 cells present, a stage is attained to which the status of a gastrula must be conceded; for it consists of a central clump of endoderm, out of which mid-gut epithelium will develop, and an outer layer of cells which may justly be considered as ectoderm. It is true that neither blastopore nor archenteron are present; but neither of these is a constant feature of the gastrula of *Peripatus*. The gastrula of *Pauropus* is, nevertheless, a much-modified gastrula; its ectodermal cells are fully as rich in yolk as are the endodermal cells, and their nuclei, in anticipation of the formation of the blastoderm, are already located at the surface. Eventually by the breakdown of the walls of the ectoderm cells and the accumulation of a surface layer of cytoplasm the blastoderm phase is entered upon, and cleav-age thereafter becomes a purely superficial cleavage on the outside of an unorganized mass of yolk. The blastoderm then becomes demarcated into a germ-band and a provisional body-wall. The mesoderm forms by separation of cells from the germ-band, and not from a blastoporal area, as in *Peripatus*. It is evident that the blastoderm must be a post-gastrula phase, and is not represented in the development of *Peripatus* (in the heavily yolked eggs of *P. novae-zelandiae* Sheldon refers to a 'blastoderm'; this is, however, a pre-gastrula phase, and is not comparable with the post-gastrula blastoderm of *Pauropus*).

In no insect and in no myriapod with the possible exception of *Scolopendra* is there such clear evidence of the presence of a gastrula as in *Pauropus*, for in one way or another the egg passes directly into the blastoderm phase. The egg of Symphyla shows, at the blastoderm stage, a remarkable resemblance to that of *Pauropus*, for the 'yolk-cells' enclosed by the blastoderm are not com-pletely specialized vitellophages, but survive, for the greater part, to form fat-body and mid-gut epithelium; the mesoderm also arises, as in *Pauropus*, by separation of cells from the germ-band. Judging by Heathcote's (1886) fragmentary description of the development of *Julus terrestris*, the yolk-cells

of Diplopoda are, like those of *Pauropus* and Symphyla, partly used in the formation of adult tissues, for the fat-body seems to arise from them. Heathcote derived the mid-gut also from a central core of yolk-cells, but all other authors (Metchnikoff, 1874; Cholodkowsky, 1895; Lignau, 1911; Pflugfelder, 1932) are agreed that it takes its origin from the germ-band itself, either in association with the stomodaeum alone, or with the latter and the proctodaeum, the two Anlagen then growing towards each other through the yolk. In Diplopoda the mesoderm arises by separation of cells from the germ-band.

In *Scolopendra* alone among myriapods do we find a form of development which is strangely reminiscent of that found in the heavily yolked species of *Peripatus*. According to Heymons' (1901) account, most of the cleavage-nuclei move from the middle of the yolk towards the periphery, where the blastoderm phase then ensues, only a few of the nuclei remaining behind in the yolk as nuclei of vitellophages. From a small area in the blastoderm (cumulus primitivus) more vitellophages then enter the yolk. This is followed by the separation of endoderm cells over the lower half of the blastoderm, these cells being applied, as mid-gut Anlage, to the outer surface of the yolk. Most of the mesoderm arises by proliferation from the cumulus primitivus of two forwardly moving bands of cells, and here we are strongly reminded of the formation of the mesoderm in *Peripatus* from a blastopore. It would seem that the 'blastoderm' of *Scolopendra* is a blastula, comparable with the 'blastoderm' of some heavily yolked species of *Peripatus*, and that it is not the equivalent of the post-gastrula blastoderm of *Pauropus*.

We turn now to the thorny question of the interpretation of the blastoderm of insect embryos. The conviction that a gastrula, with its ancestral germ-layers, must at all costs be revealed in the development of these arthropods seems to have introduced little but confusion into the subject.

Haeckel (1877) regarded the blastoderm as a modified blastula. Supposedly basing his interpretation on Kowalewsky's description of the development of *Hydrophilus*, Haeckel identified the gastrula with the immediately following stage in which the ventral groove invaginated. Yet Kowalewsky had observed that the invaginated 'lower layer' was not endoderm, nor its cavity an archenteron.

Balfour (1880) saw the error of this interpretation. Though accepting the view, then prevalent, that the mid-gut arose from yolk-cells, he denied the existence of a gastrula in the development of insects on the ground that the formation of the yolk-cells could not be reduced to an invagination process.

In a later work Kowalewsky (1886) reintroduced Haeckel's gastrula, though in a modified form. He had observed the formation of the mid-gut, not from yolk-cells, but from the anterior and posterior 'endoderm rudiments' which themselves form the anterior and posterior tips of the 'lower layer' that invaginates along the ventral groove. For him the invaginating groove was an elongate blastopore, with the endoderm confined to its ends, and with a long

strip of mesoderm intervening. This view has found favour with many writers, the more so since the divided blastopore of *Peripatus* seemed to afford a possible clue to the origin of the elongate 'blastopore' of insects.

Kowalewsky's gastrula met its first important criticism in Heymons' great work on the development of Dermaptera and Orthoptera (1895). In these primitive insects the mid-gut was found to arise by ingrowth of cells from the stomodaeum and proctodaeum. In some species the ventral groove was not even present, but the mesoderm arose by separation of cells from the germ-band. This latter type of mesoderm formation is common to all myriapods hitherto examined except *Scolopendra*, as well as to the Collembola, *Lepisma, Campodea, Eutermes*, and many Orthoptera. The ventral groove, far from being a modified blastopore, is thus found to have originated among the Insecta themselves.

Heymons' observation that the mid-gut epithelium developed from stomo-daeum and proctodaeum was in such obvious conflict with current notions of morphogenesis, based on an acceptance of Haeckel's 'Gastraea theory', that it encountered much opposition—Nusbaum and Fulinsky (1906), Hirschler (1909), MacBride (1914), and others. Yet it has been repeatedly confirmed, and especially by more recent writers, for insects of different orders—*Diacrisia* (Lepidoptera), Johannsen (1929); *Calandra* (Coleoptera), Mansour (1927), Tiegs and Murray (1938); *Locusta*, Roonwal (1936-7); and *Pteronarcys* (Perlaria), Miller (1940)—and seems to hold also for some Diplopoda.

Applying germ-layer terminology, Heymons concluded that the mid-gut in Orthoptera was 'of ectodermal nature'. It is not necessary to consider the validity of this proposition; it will suffice to agree that it cannot be endoder-mal. For the term 'endoderm' must be restricted to the mid-gut Anlage when this forms the inner layer to a gastrula, unless it is to become a superfluous alternative to 'mid-gut-epithelium' in general. Consistently with germ-layer principles, the need now arose for identifying endoderm in the embryo. In this dilemma Heymons, and many others following him, have turned to the yolk-cells. The development of *Campodea* and *Lepisma* seemed to lend countenance to this view, for in these insects the mid-gut arises from cells which, migrating from the blastoderm into the yolk, assumed control over the yolk itself.

In *Pauropus* and *Hanseniella* (Symphyla) also the mid-gut is derived from yolk-cells; yet it is to be observed that in these myriapods most of the 'yolk-cells' are used, not in the formation of mid-gut epithelium, but of fat-body, a tissue which in insects is of mesodermal origin. In *Scolopendra*, as Heymons has shown, true yolk-cells (vitellophages) and endoderm are simultaneously present; had the mid-gut in this myriapod developed instead from stomo-daeum and proctodaeum, as it does in many insects, then assuredly the vitello-phages would have been invoked to uphold the germ-layer principle.

The validity of identifying yolk-cells, or any other cells, with endoderm, when these do not give origin to the mid-gut, involves the difficult theoretical problem of applying the principle of homology to an undifferentiated region

within an embryo. The concept of homology has emerged from a comparison of organs. Spemann (1915), in a lucid discussion on the propriety of applying this concept to unorganized regions of an embryo, writes: 'Homologizing is only possible after the formation of Anlagen, i.e. at a developmental period when the individual parts of the germ have become differentiated, if not in their outward appearance, at least in their developmental tendency.' To homologize yolk-cells with endoderm therefore implies a belief that they are the vestige of a discarded mid-gut, and that the 'ectodermal' mid-gut is a completely new organ.[1] Such are the difficulties, and indeed absurdities, which follow any attempt to explain away discrepancies in an outworn theory of development that did not contemplate such facts.

In the development of *Pauropus* we now seem to have a clue to the interpretation of the blastoderm phase; it is a stage which succeeds a transitory gastrula, and is not, therefore, comparable with the 'blastoderm' of a heavily yolked *Peripatus* egg, which is a modified blastula. In other myriapods and in insects the gastrula has become superseded altogether, and the segmenting egg then passes directly into the blastoderm phase. It is a distinctive feature of these ontogenies that the mesoderm develops by separation of cells, either diffusely, or bilaterally, along the germ-band, or by the more specialized process of invagination along a ventral groove, and not, as in *Peripatus*, from a blastoporal region ('primitive streak'); in *Scolopendra* alone does a more primitive mode of mesoderm formation, by cell-proliferation from a 'cumulus primitivus' (primitive streak), seem to have largely survived.

The function of the blastoderm is evidently to eliminate the impeding action of yolk on morphogenetic processes in the egg. In Pauropoda, Symphyla, many Collembola and Diplopoda, the adaptation to yolk-accumulation is imperfect, for it is certainly surprising to observe the interior of the egg reverting to an unsegmented condition after the initial phase of apparently futile total cleavage. In *Pauropus* these early cleavages even permit the formation of a gastrula. In all these primitive groups, moreover, yolk-laden cells in the interior of the egg, after playing an initial role as vitellophages, become organized into specific tissues of the adult. In insects (except Collembola and a few parasitic forms) there is no segmentation of the egg attending cleavage of the nuclei, and the egg passes directly into the blastoderm condition. Yolk-cells (except in *Lepisma* and *Campodea*) are now purely vitellophages, and the tissues which develop out of yolk-cells in the more primitive groups are now organized from other sources. It is evident that as this specialized type of development has evolved, all trace of a gastrula, or of a gastrulation process, as still exemplified in *Pauropus*, has been lost.

Vertebrate embryos, confronted with much greater problems of yolk-accumulation, have, like those of the heavily yolked species of *Peripatus*, preserved to a surprising degree the primitive type of development of their ancestors; for the early developmental processes of vertebrates, however

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[1] One writer (Lecaillon 1898) has even seriously proposed thus to avoid conflict with the germ-layer theory.

much obscured, can still be reduced to a gastrulation process, and do not show such remarkable departures from the primitive as the developing insect reveals.

6. Development of the External Characters of the Embryo

(i) *Early Development of the Germ-band, and the Formation of Appendages.* By about the end of the fifth day the head-lobes have appeared (fig. 23, Pl. 2). In the region of these lobes the germ-band is at its greatest width. The lobes are not yet very pronounced; they are, however, distinctly paired, for there is a deep indentation between them at the anterior tip of the germ-band. By this time a small elongate stomodaeal orifice has arisen, and the proctodaeum is now also in course of formation.

During the sixth day the antennae begin to form. In entire embryos they may be seen as a pair of just perceptible backwardly directed elevations on the head-lobes, a little behind the stomodaeum, their outline being most readily distinguishable along their inferior margin (fig. 24 A and B, Pl. 2). There are no intersegmental grooves delimiting the antennary segment, and, indeed, throughout the length of the germ-band such grooves do not appear until the embryo is in an advanced state of development.

By the seventh day the embryo has assumed the form shown in fig. 25 A and B, Pl. 2. The stomodaeal opening is still conspicuous. The head-lobes have now much enlarged (cf. the relative position of the stomodaeum in figs. 23, 24 B, and 25, B, Pl. 2), and show the orifices of a pair of deep invaginations, which are the Anlagen of the posterior lobes of the protocerebral ganglia. The antennae are considerably enlarged and more sharply defined; their bases now lie on about a level with the stomodaeum. Behind the antennae evidence of two new segments has appeared, namely the pre-mandibular and mandibular, but neither is demarcated by transverse grooves. The former occupies a large area of the germ-band behind the antennae, but at no time does it possess even a rudiment of appendages. The mandibular segment, on the other hand, is made evident externally by its pair of large mandibles; they are still little more than gentle elevations of the surface of the germ-band, the alignment of nuclei around their margin accentuating their outline.

Before the end of the seventh day several additional segments have become defined. An embryo at this stage of development is shown in fig. 26 A and B, Pl. 2. The stomodaeal opening has now become a wide transverse cleft. The antennae are much more distinct. The mandibles have also become more sharply defined, and the first outlines of the maxillae are distinguishable behind them. It is to be observed that both these appendages are, from the first, directed medially. The first leg-bearing (second abdominal) segment is now also becoming recognizable by the presence of its appendages, which arise as gentle protuberances from the germ-band, farther from the mid-line than the gnathal appendages, and at first much smaller than these. Between the maxillary and first leg-bearing segment is the collum (post-maxillary) segment, but it does not bear the Anlagen of any appendages; it does not become part of the head, but forms the first of the abdominal segments. By this time

certain 'ventral organs' (cf. section 13) have appeared, and these afford a use-
ful guide to the subsequent displacement of the head-segments; they do not
protrude beyond the surface, but are recognizable in stained whole embryos
by the peculiar radiating orientation of their nuclei. They are already present
in the pre-mandibular, mandibular, and maxillary segments (fig. 26 B, Pl. 2).

A rather more advanced embryo, aged about 8 days, is shown in fig. 27 A
and B, Pl. 2. The first legs have considerably enlarged, and the second have
also appeared. Behind these a pair of just perceptible swellings on the germ-
band indicates the formation of the third legs. On the head the orifices of the
invaginating posterior lobes of the protocerebral ganglia, which have hitherto
been conspicuous, are no longer present. The maxillae are now sharply
defined. The only other noteworthy change concerns the beginning of forma-
tion of the pre-oral cavity ('mouth-cavity'), a description of which is given
below (see Development of Head).

I have already referred to the absence of any intersegmental grooves what-
ever in the early embryo. By the eighth day, however, a rather vague indica-
tion of some of the segments has appeared, in the form of slight encroachments
from the margin of the germ-band on to the provisional body-wall, and be-
tween them perceptible grooves are appearing. These grooves, it must be
stressed, are confined to the lateral margin of the germ-band, and do not
extend on to its lower surface. They are visible in the embryo shown in fig.
27 A, Pl. 2, those that define the collum and second abdominal (first leg-bearing)
segments being the first to appear.

Thus far the distinction between the germ-band and the thin provisional
body-wall has been preserved. In the latter, however, mitoses are not infre-
quently seen, and there is not that paucity of cells that we find commonly in
the provisional body-wall of other myriapods and insects. In some embryos
the nuclei are even closely crowded (e.g. fig. 26 A, Pl. 2), but there is much
variation in this respect. In all cases, however, it is a thin membrane, and the
yolk is always easily visible through it.

(ii) *The Advanced Embryo.* During the eighth day the first embryonic cuticle
begins to form. It is rather thicker than the blastodermic cuticle. Its most
striking feature is a band of setae, which lies transversely across the head.
The individual setae that compose the band are relatively long, stout, and
sharp, and are disposed in three transverse rows, there being eleven in the
first, ten in the last, and a short row of four between them. In 9-day embryos
these setae can readily be seen through the transparent egg-membranes
lying against the surface of the embryo. During the tenth day they become
erect, and thereby tear a wide transverse rent in the overlying blastodermic
cuticle and chorion, through which they now protrude (Text-fig. 1). Ad-
vanced eggs can always readily be identified by these protruding setae; the
egg is, moreover, no longer spherical, but has begun to lengthen.

A few days before the embryo is due to emerge from the egg a second
embryonic cuticle is formed. This is the 'pupoid' cuticle. It also is adorned
with setae; but unlike those of the first cuticle, they are mostly very long,

curved, and delicate, and are distributed over much of its dorsal and lateral surfaces. As the second cuticle develops, the tear in the egg-membranes slowly enlarges. On about the twelfth or thirteenth day it widens, a rent then appearing also in the embryonic cuticle, and so the pupa is slowly liberated (Text-fig. 3). The discarded chorion, blastodermic, and embryonic cuticles remain adhering to its under surface.

In describing the changes which meantime have taken place in the external form of the embryo, it will be convenient to consider the abdomen and head separately.
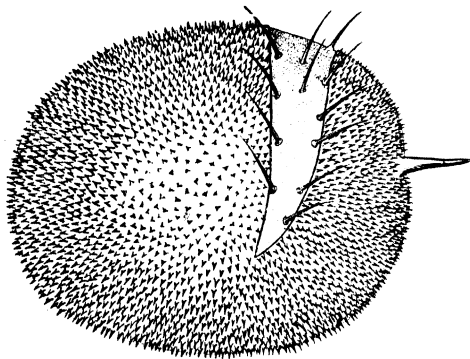
TEXT-FIG. 1. Egg, showing initial rupture of chorion; cutting setae of embryonic cuticle protruding. Anterior end to right.

A. *The Abdomen.* During the ninth day the formation of the definitive body-wall is in active progress (fig. 28 A, Pl. 3). All along the margin of the germ-band cells are beginning to spread upward, and the intersegmental lines delimiting the collum and second abdominal segment are now quite clear, owing, in a measure, to the fusiform character of their cells and of their nuclei.

In the advanced embryo shown in fig. 29 A, Pl. 3 (aged about 10 days), the body-wall is almost complete. The intersegmental lines have now deepened into grooves, the groove behind the first leg being an exceptionally deep cleft. The grooves completely encircle the embryo, spreading now even on to its ventral surface; it is, however, on the dorsal surface that they attain their greatest depth. This dorsal accentuation of the clefts is evidently a consequence of the transverse folding to which the epidermis is there subjected, for the embryo is still in a somewhat dorsally flexed condition.

The segments which have thus become demarcated are the collum and second to fourth abdominal segments. The collum segment is markedly wedge-shaped, its tergal portion being reduced to a narrow strip of epidermis. Some time before hatching, its sternal wall enlarges almost to the size of the segment that succeeds it (cf. figs. 29 B and 30 A, Pl. 3). The fourth abdominal segment, which bears the third pair of legs, is also a wedge-shaped segment,

for the intersegmental line that delimits it behind merges, toward the dorsal surface of the embryo, with the intersegmental line behind the preceding segment (figs. 29 A, 31, Pl. 3). The pleural areas of the collum and three leg-bearing segments are thin, and through them the yolk is still readily seen. These areas remain permanently thin, for there are no muscles attached to them.

It is in these advanced embryos that we see the first indication of the inter-segmental line that delimits the fifth abdominal from the anal segment. If the embryo is turned on its end, so that it can be viewed from behind, the limits of the new segments are already seen clearly outlined by a ring of fusiform cells, even though an actual groove is not yet present. Near the middle of the last segment is the anus (fig. 31, Pl. 3).

On the lateral part of the tergal wall of the fifth and third abdominal segments there may be seen, in all embryos after the tenth day, a pair of small but conspicuous patches of rather deeply staining cells; they are the developing sensory setae (trichobothria); figs. 29 A, 31, Pl. 3.

On the ninth day the legs begin to elongate more actively. Unlike the mouth-appendages, they are, from the beginning, outwardly directed. They do not show any localized growing zone, for mitoses appear at random along their length. They soon become bent on themselves. Thereafter the development of the first pair, hitherto much the largest, is retarded, the second and third even outstripping the first a little. In the advanced embryo the legs become narrower, especially towards their tips, where they bend well under the embryo. Only at about the time of liberation of the pupa does the first sign of their segments appear.

On the collum segment legs are absent. On the floor of this segment there are present a pair of large swellings, each bearing a minute medially directed conical papilla (Text-figs. 2 B, 24 B). Following Latzel (1884) it is customary to regard the latter as the vestigial appendages of the collum segment. The swellings on the floor of the segment which bear these problematical organs do not appear till about the tenth day, and show no obvious resemblance to limb-Anlagen (fig. 30 A, Pl. 3). They are referred to again in section 14 (v).

B. *The Head.* (*a*) Morphology of adult head (Text-fig. 2). The descriptions of the head-capsule that have passed into current authoritative works on the Pauropoda are inadequate, and incorrect on points of importance; for, from its minuteness and the complexity of its structure, this is a most difficult part to examine. The following account is given to facilitate the embryological description which follows; but a knowledge of the structure of the head is also of much importance for the assessment of affinities.

The head, which is carried in a depressed (hypognathous) attitude, appears somewhat triangular when viewed from above. Four sutures run across its dorsal surface, which is thereby divided into five areas (Text-fig. 2 A). I shall speak of these as the clypeus, antennal area, frons, vertex, and occiput; the terms have been chosen for convenience, and no homology with any similarly named parts of an insect head is implied. The line between clypeus and
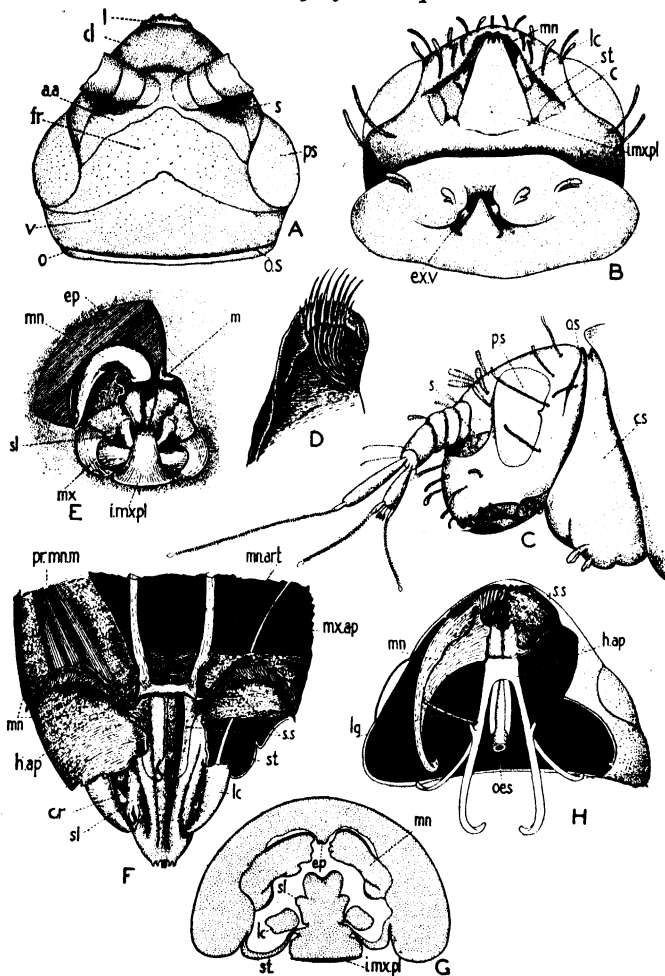
TEXT-FIG. 2. The Head.

A. Dorsal view; setae not drawn. B. Ventral view; collum segment included in drawing; left maxilla has been drawn out from under intermaxillary plate. C. Lateral view; collum segment, and part of succeeding segment, included in drawing. D. Tip of right mandible, viewed from below. E. View into pre-oral cavity (diagrammatic). F. View from above, to show floor of pre-oral cavity; part of the right mandible and most of the left removed; cavity of left maxilla partly exposed; part of interior of head-capsule also shown. G. Transsection through head, taken at level of bases of superlinguae. H. Ventral view of head, showing suspensory sclerite, hypopharyngeal apophyses, and right mandible.

Lettering. *a.a* antennal area; *c* cardo of maxilla; *cl* clypeus; *c.r* chitinous ridge against which maxilla rests; *c.s* collum segment; *ep* epipharynx; *ex.v* exsertile vesicle; *fr* frons; *h.ap* hypopharyngeal apophysis; *i.mx.pl* intermaxillary plate; *l* 'labrum'; *lc* lacinia of maxilla; *lg* fibrous ligament between mandibular apodeme and hypopharyngeal apophysis; *m* mouth; *mn* mandible; *mn.art* articulation of mandible with hypopharyngeal apophysis; *mx* maxilla; *mx.ap* maxillary apodeme; *o* occiput; *oes* oesophagus; *o.s* occipital suture; *pr.mn.m* protractor muscle of mandible; *ps* pseudoculus; *s* sclerite for attachment of dorso-lateral longitudinal muscle of head; *sl* superlingua; *s.s* suspensorial sclerite; *st* stipes of maxilla; *v* vertex.

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antennal area is a deep recurving fissure; that between the vertex and occiput (occipital suture) forms a strong phragma for the attachment of tergal muscles. The other two sutural lines are merely thin seams in the dorsal chitin, and are easily overlooked. The occipital suture appears, from its development, to be the mandibulo-maxillary intersegmental line, the short occiput to the rear of it being the reduced tergal wall of the maxillary segment; whether the other sutures have any segmental significance cannot be determined. The chitin of the clypeus becomes thinned out, at its tip, to form a very inconspicuous 'labral lobe', fringed with tiny curved spines, and demarcated by a groove from the clypeus. Along its margins the latter is, as Silvestri (1902) has already shown, folded in beneath the mandibles, which are therefore shut in except at their tips (Text-fig. 2 B).

On the sides of the head are the large bulging 'pseudoculi'. These are described in section 14 (iii).

Immediately behind the bases of the antennae, in the position occupied by the post-antennary organs of Tömösvary in Symphyla, are a pair of small rectangular sclerites (Text-fig. 2 A, C); they do not, however, represent such an organ, but are thickenings of the chitin, to receive the insertion of the dorso-lateral muscles of the head (see section 15).

The bifurcated antennae (Text-fig. 2 C) arise close together on the antennal area; adequate descriptions of these remarkably specialized appendages have already been given by Lubbock, Latzel, Kenyon, Remy, Silvestri, and others.

The examination of the mandibles presents considerable difficulty, for the conformation of their chitin is complex, and they are, moreover, closed in by the inturned folds of the clypeus. They consist each of an unsegmented piece of chitin, whose base is prolonged into an apodeme, a long, curved blade of chitin, which extends far back into the cavity of the head (Text-fig. 2 H). The apodemes are attached each by two fibrous ligaments, a median to the hypopharyngeal apophysis (Text-figs. 2 H, and 12), and a lateral to the wall of the head just to the rear of the pseudoculus (Text-fig. 21 A). Each mandible is articulated with the hypopharyngeal apophysis (Text-fig. 2 F, H). Only the anterior quarter of the mandible is free to operate within the pre-oral cavity, and here it displays a most unusual structure: on its superior surface the chitin is thick and firm, but on its inferior surface it is thin and has the form of a shallow groove. Along this groove run fine ridges of chitin, about seven in number, and these are prolonged beyond its tip to form a comb of seven delicate curved blades, beyond which is a second row of smaller blades (Text-fig. 2 D, H). The two grooved surfaces of the mandible co-operate with an epipharyngeal ridge to form the upper half of a tube, the lower half of which is completed by the grooved floor of the pre-oral cavity, against which a flange from the mandibles fits; and the whole structure is evidently a contrivance for drawing into the mouth semifluid food scraped away by the fine terminal blades of the mandibles. As other authors have already observed, the intestinal content has a fluid consistency. Nevertheless, animal remains are occasionally met with. For their possible origin see section 9 (a).

Below the mandibles is a single pair of maxillae, and between them the intermaxillary plate (Text-fig. 2 B). The latter is a simple triangular piece of chitin, bearing at its tip several short, blunt spines. The maxillae are composed of two parts, distinguishable as stipes and lacinia. There is no galea or palp. The lacinia is an elongate tapering piece of chitin, with a slender apodeme that extends far into the head, where it becomes attached to one of the ligaments of the mandibular apodeme. The lacinia is partly covered, from below, by the intermaxillary plate, and rubs against an inturned chitinous process from the latter (Text-fig. 2 F). The stipes is a short, semi-cylindrical sclerite devoid, as usual, of an inner wall, and forming therefore part of the head-wall itself. Behind the latter is another, and smaller, sclerite, the equivalent, it would seem, of the cardo; but, unlike the other two parts of the maxilla, it has no muscle-attachments and should therefore probably not be reckoned as part of the appendage itself, but rather as a 'pleurite'.

The intermaxillary plate, which is the sternite of the maxillary segment, does not itself constitute the floor of the pre-oral cavity, the latter being formed mainly out of the inturned sternum of the mandibular segment, the whole protruding structure being a kind of 'lower lip'. Along it runs the gradually widening and deepening groove above alluded to, which leads behind into the mouth (Text-fig. 2 E, F). To the sides are a pair of appendage-like structures, ending in several minute teeth, and already referred to by Silvestri (1902) as the galeae of the intermaxillary plate; actually, however, they arise from within the pre-oral cavity (Text-fig. 2 G), with the floor of which they are flexibly articulated, but they do not seem to be provided with muscles. They are a product of the mandibular segment, and seem therefore to be the equivalent of the superlinguae.

A pair of unusually complex suspensorial sclerites for the attachment of the hypopharyngeal apophyses is present (Text-fig. 2 F, H). They occupy part of the hinder wall of the pre-oral cavity, above the bases of the mandibles, where there are attached to them the protractor muscles of these appendages (see section 15). From here they extend down below the mouth, and then bend forward to form a support for the floor of the pre-oral cavity. They are not associated with the superlinguae.

The hypopharyngeal apophyses (Text-fig. 2H) are a pair of complex chitinous structures, from which many of the sternal muscles of the appendages take origin. (Ferris (1942) has justly criticized the term 'hypopharyngeal apophyses' which I have retained here in deference to its use in recent standard works on insect morphology (Snodgrass, Weber). The apophyses do not arise from the hypopharynx, even when that term is used, in the wider sense, to include the superlinguae.) Immediately behind their point of attachment to the suspensorial sclerites they form a chitinous ring round the oesophagus, from which two sheaths extend back some distance along the latter. The principal arms of the apophyses pass back into the collum segment, where they bend round the tritocerebral ganglion to provide attachment for the large dilator muscles of the oesophagus (Text-fig. 12). In addition

there is present a pair of smaller arms, which bend upwards and become attached by fibrous tissue to the occipital suture; these act as braces for the whole structure, and there are no muscles attached to them. The development of the apophyses is described in section 14 (vi).

(b) Development of the head. The principal theoretical problem in the developing head is the determination of its segments, and the identification of these in the completed head-capsule. Most morphologists agree that the procephalon has arisen by the welding together of an acron (prostomium) and three segments, viz.: the pre-antennary, antennary, and pre-mandibular. The principal evidence for this is provided by Heymons' (1901) study on *Scolopendra*, in the embryo of which the constituent segments of the procephalon are identifiable, to an unusually complete degree, by their ganglia, mesodermal somites, and intersegmental grooves. The hope that so primitive a myriapod as *Pauropus* might yield still more complete evidence on this point has not been fulfilled, for intersegmental lines are here markedly suppressed throughout the whole length of the germ-band. The chief external guide to the segments must therefore be the appendages and the 'ventral organs'. The latter are well developed on the antennary and pre-mandibular segments, those of the pre-antennary segment being diminutive; appendages occur on the antennary segment alone. The pre-antennary segment and acron are not externally distinguishable.

In the gnathocephalon two segments only are present, the mandibular and maxillary; there is no second maxillary segment. Each has a pair of large appendages and a pair of 'ventral organs'. An intersegmental line demarcates these two segments from one another, but, like the other intersegmental lines, it does not appear until late in the development of the embryo.

By the sixth day the antennae have begun to form. They are, at first, merely gentle swellings of the epidermis, and, as usual in myriapods and insects, lie post-orally (fig. 24 B, Pl. 2). By the seventh day they are more distinct, and have moved up almost into line with the stomodaeum; the mandibles have now also begun to form, and the pre-mandibular segment is distinguishable (fig. 25 B, Pl. 2). In the late 7-day embryo, as shown in fig. 26 B, Pl. 2, the bases of the antennae lie on a level with the stomodaeum, which is now a wide slit; the maxillae are beginning to form, and the pre-mandibular, mandibular, and maxillary 'ventral organs' are distinguishable. The impression obtained from this series of embryos is that a post-oral antennary segment is in process of curving forward round the stomodaeal opening.

During the eighth day the pre-oral cavity begins to form. The head is still very large and occupies most of the anterior half of the egg (fig. 27 A, Pl. 2). The mandibles have moved nearer to the mouth, and the pre-mandibular segment therefore appears of diminished size. This is due to the fact that the median sternal portion of the segment is beginning to invaginate in the direction of the former stomodaeal opening, pushing the latter more deeply into the head. The new cavity which is thus forming is the pre-oral cavity, and its floor consists of pre-mandibular epidermis (fig. 27 B, Pl. 2). At its corners

are situated the 'ventral organs' of the pre-mandibular segment. If the head is turned on its side and examined in optical section the pre-mandibular (tritocerebral) ganglia, hitherto post-oral in position, are now seen to the side of the stomodaeum. It is therefore clear that the pre-mandibular segment, like the antennary, has now also begun to curve forward from behind the original stomodaeal opening.

In the 9-day embryo shown in fig. 28 A and B, Pl. 3, the antennae have moved into a pre-oral position, and the mandibles now lie in line with the pre-oral cavity. The median sternal portion of the pre-mandibular epidermis has completely disappeared from the surface; but if the head is turned on its side, it can be seen, in optical section, forming the floor of the pre-oral cavity. Comparison with fig. 27 B, Pl. 2, shows, at the same time, that the more lateral part of the pre-mandibular epidermis has moved into a pre-oral position, and occupies an area, with unidentifiable outline, median to the base of each antenna; this epidermis later plays a large part in the formation of the clypeus, and it is therefore evident that, as in Symphyla, this part of the head-capsule must be largely of pre-mandibular origin. (Both in insects and myriapods the innervation of the clypeus and labrum from the tritocerebrum has long been known (Saint Remy, 1887; Viallanes, 1887; Holmgren, 1916), and there can be little doubt of an extensive participation of the pre-mandibular segment in the formation of these parts in other myriapods and insects.) The sternum of the mandibular segment is now a conspicuous triangular structure forming a lower lip to the pre-oral cavity (fig. 28 B, Pl. 3); already in the earlier embryo shown in fig 27 B, Pl. 2, this structure is recognizable by the arrangement of its nuclei.

In the 10-day embryo shown in fig. 29 A and B, Pl. 3, the antennae have grown longer and more slender, and their bases have begun to approach one another on the anterior wall of the head. There can no longer be any doubt as to the reality of the displacement and deformation to which the remarkable antennary segment is subjected. The clypeus has now started to fold down over the bases of the mandibles, and this has the effect of greatly widening the pre-oral cavity and of enclosing the mandibles within it. The mandibular sternum still protrudes as a kind of lower lip to the pre-oral cavity. The head has, by this time, already begun to diminish in relative size (cf. fig. 27 A, Pl. 2, and fig. 29 A, Pl. 3).

Enclosure of the mandibles within the pre-oral cavity is not complete until shortly before the embryo leaves the egg as a 'pupa'. An embryo at this stage of development is shown in fig. 30 A and B, Pl. 3. The maxillae are now conical in form, and protrude towards the tip of the clypeus. Between the maxillae is the intermaxillary plate. The mandibular sternum does not form a part of it, but it is derived entirely from the maxillary sternum. From the structure of the head in the earlier embryos shown in figs. 28 B and 29 B, Pl. 3, the participation of the mandibular sternum in its formation might have been expected; there is, however, no evidence for this, and indeed, if the embryo be turned on end to admit a view into the pre-oral cavity, the inturned mandibular

sternum may be seen forming the floor of that cavity between the mandibles (fig. 30 B, Pl. 3). For comparison with the gnathochilarium of diplopods it is also of importance to observe that the collum segment does not contribute to the formation of the intermaxillary plate; for though the intersegmental groove between the two segments is not very deep, it is sufficiently clear to define precisely the limits of the two segments. (Some further observations on the development of the intermaxillary plate are given in section 10 (iv).) The antennae have now grown narrower and longer, and at their distal ends have begun to bifurcate; their bases have approached still more closely on the anterior surface of the head.

The usual difficulty of determining the limits of the gnathal segments within the head-capsule is accentuated, in *Pauropus*, by the absence of muscle indications. There is only one intersegmental line, namely, that between the mandibular and maxillary segments, and it does not appear until about the tenth day. It is then distinguishable by the fusiform character of its cells, but can at first be seen extending up for only a little distance from between the bases of the mandibles and maxillae (fig. 29 A, Pl. 3). In pupae (figs. 32 A, 33 A, Pl. 3) it becomes more prominent, and now encircles the head, developing above into a pronounced groove. This groove remains as the occipital suture in the chitinized head-capsule, but does not extend the full distance to the bases of the appendages.

The groove demarcating the clypeus from the antennal area is present from the tenth day onwards (fig. 29 B, Pl. 3).

The superlinguae do not make their appearance till after the pupa has formed; they are, for example, not present in the advanced embryo shown in fig. 30 B, Pl. 3. They arise as a pair of outgrowths from the floor of the pre-oral cavity, being the product of the mandibular epidermis (Text-fig. 9).

A feature of the very advanced embryo is the pronounced deepening of the intersegmental grooves behind the first abdominal segment, and the formation of several transverse folds that distort the body-wall, especially on the head. It is evident that the epidermis of the advanced embryo has enlarged to a degree where it can no longer be accommodated without distortion within the old embryonic cuticle. Presumably in consequence of absorption of moisture from without, the surface distortions now gradually even out, the embryo enlarges, and so slowly forces its way from the egg to enter the 'pupoid' phase.

(iii) *The 'pupoid' phase.* The pupa (Text-fig. 3) is a smooth, white object, measuring 0·2 mm. in length. It is quite immobile. It is rather narrower in front than behind. Minute oral and anal apertures are present, the chitin being inflected through them to form a lining for the fore-gut and rectum. The pupal sheath shows the impress, from within, of the bifurcated tips of the antennae, and of the tips of the first and second, but not third, legs. It is clothed with setae, most of which lie in transverse rows and are long, slender, and curved, though there are also a few that are relatively short and stout.

The appearance of the stained embryo, seen through the transparent pupal sheath, is shown in fig. 32 A, Pl. 3. The head has assumed almost its adult

form. On the antennae the three flagella are beginning to grow out (fig. 32 B, Pl. 3). The collum and first leg-bearing segments have assumed almost their definitive form, but the hinder segments are still pressed against one another and it is due to the release from this that the subsequent elongation of the larva, after leaving the pupa, is due. The tergal wall of the second and third segments is strongly developed, but that of the following segment is reduced to a narrow strip of epidermis, being, as it were, wedged in between the third and fifth segments. The anal segment is now sharply demarcated from the
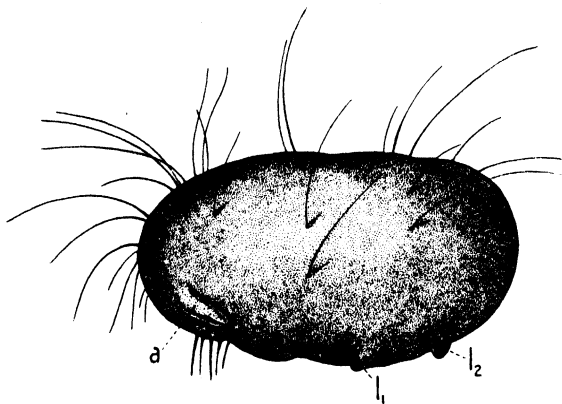
TEXT-FIG. 3. The pupa.
Lettering. a antenna; l_1, l_2 first and second legs.

fifth. The legs have become further enlarged and bent under the ventral surface of the embryo, and are beginning to show signs of segmentation (fig. 32 C, Pl. 3). Only the five segments which form the segments of the mature limb are distinguishable, there being no indication of a 'sub-coxa' postulated by some morphologists.

In the pupa the flagella of the antennae elongate rapidly (figs. 33 A and B, Pl. 3) and the globulus is now also visible. There are no nuclei in the flagella. The latter are protoplasmic prolongations from cells at the tips of the rami, and several cells seem to co-operate to form them.

Chitinization does not begin until about the end of the second day, and thereafter the various setae and specialized 'hairs' of the larva become visible beneath the pupal sheath. Special importance attaches to the development of the second and third tergites. The former is entirely the product of the tergal wall of the third (second leg-bearing) segment, and the reduced tergal wall of the succeeding segment contributes nothing to it. Similarly the tergite of the fifth abdominal segment will become the third in the adult animal, and it, also, is the product of a single segment, the future sixth abdominal segment (which is related to the fifth as the fourth is to the third—see Text-fig. 24 A) being still only in course of formation. This solves the problem of

the supposed 'diplotergites' of *Pauropus*; they are not 'diplotergites', but derivatives of a single segment, and each tergite-bearing segment (except the first) is followed by an 'intercalated' segment with reduced tergal wall, and without a tergal shield (see further, Post-emb. Dev., sections 1, 2).

After 3 or 4 days a split appears along the dorsal surface of the pupa, and the larva is set free.
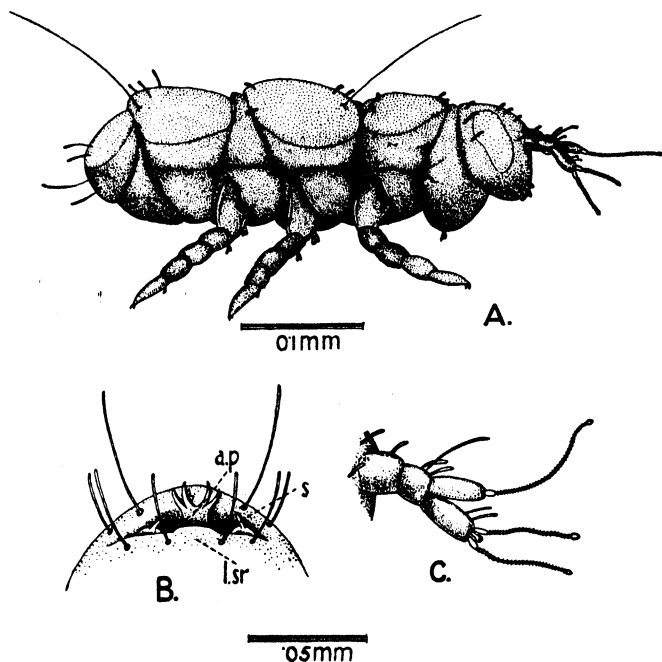
TEXT-FIG. 4. First Instar Larva.
A. Entire larva. B. Hinder end. C. Antenna.
Lettering. *a.p* anal plate; *l.sr* lamina supranalis; *s* style.

(iv) *The First Instar Larva* (Text-fig. 4). This is a small, white, actively moving creature, measuring about 0·25 mm. in length.

The head of the larva possesses most of the characters of the adult animal. On the antenna the globulus and three flagella are present; there are, however, only two basal segments in the antenna, and the number of rings in the flagella is only about a third of the adult number (Text-fig. 4 C). The mandibles and maxillae have assumed their adult features. The pseudoculi are present.

The collum segment is wedge-shaped, and is without a tergal shield. On the second segment a shield is present. There is a similar shield on the following segment, and this bears a pair of long sensory setae (trichobothria), the specialized epidermal cells from which these arise being already evident in

10-day embryos (fig. 29 A, Pl. 3). The fourth segment is without a shield; in extended larvae its reduced tergal wall is readily seen (Text-fig. 4 A), but if the larva is contracted it is withdrawn beneath the second tergal shield, which thereby acquires the misleading appearance of a diplotergite. The third shield, that of the fifth abdominal segment, bears, like the second, a pair of long tactile setae; this shield will remain as the third shield in the next and later instars, the new segments being generated from the anal segment. There is a fourth tergal shield, namely, on the anal segment, but it is smaller and weaker than the three that precede it. This segment is exceptional in being provided with a sternite, the only sclerotized sternite in the abdomen.

In the first instar larva the first teloblastic (i.e. sixth abdominal) segment has already become defined. Like the fourth segment, it has a reduced tergal wall, and is wedged in behind the fifth segment. It may be seen in Text-fig. 4 A. For an account of its development, see below, Post-emb. Dev., section 2.

It is noteworthy that limb-buds for the new segments are not yet present, and in this respect *Pauropus* differs from the Symphyla, in which the first pair of legs that mature during anamorphosis is already present in the advanced embryo.

Up to the time when the embryo is entering the pupal phase, the anus has remained a transverse slit at the tip of the anal segment. But in the larva it is withdrawn from the surface (Text-fig. 4 B); it is overhung by the 'lamina supranalis', while below it is the 'lamina subanalis', but neither is demarcated by a groove from the rest of the anal segment. The lamina subanalis bears the peculiar 'anal plate' (Hansen, 1902); under the lamina supranalis, to the sides of the anus, are two papillae that bear the styles.

Each leg has five segments, the tarsi of the first instar larva being unsegmented in all the legs.

(v) *General Remarks.* In comparison with other myriapods and with insects, the external form of *Pauropus* develops along comparatively simple lines. The initial dorsal flexing of the germ-band is well known also in chilopods, many diplopods, and, amongst primitive insects, in Diplura and Collembola; but in all these forms it soon changes to complete ventral flexing, the latter being present, even from the beginning, in Symphyla and many Diplopoda. In *Pauropus* alone amongst myriapods does the slight dorsal flexure persist. The absence of embryonic membranes was to be expected; these are confined to the insects and have, indeed, evolved within that class.

Pupoid phases are of widespread occurrence amongst myriapods, though in no previously described case is there so distinctive a 'pupa' as in *Pauropus*. By a 'pupa' we mean a precociously liberated motionless embryo, with unsegmented or only partially segmented appendages, invested by a protecting cuticle, within which the embryo completes its development at the expense of the nutritive yolk. Amongst the diplopods a pupoid phase was observed by Newport as long ago as 1841, and has since been found in various members of the group. In *Strongylosoma* and *Polydesmus* (Metchnikoff, 1874) and in *Julus terrestris* (Heathcote, 1886) the 'pupa' is enclosed, as in *Pauropus*, within a specific

embryonic cuticle which already shows the rudiments of appendages. In *Julus morelettü* (Metchnikoff, 1874) the pupa is encased within a blastodermic cuticle, and this seems to hold also for *Archispirostreptus*, in which limb-buds do not protrude (Robinson, 1907). In *Glomeris*, on the other hand, a pupoid phase seems to be omitted (Hennings, 1904). Amongst the chilopods, *Geophilus* (Metchnikoff, 1875) and *Scolopendra* (Heymons, 1901) both show precocious rupture of the egg-membrane, thereby partially disclosing an embryo encased within an embryonic cuticle. Heymons, who has given a detailed account of these phases in *Scolopendra*, finds that the embryo now moults and emerges from the egg as a motionless 'embryonic stadium', beneath the cuticle of which development continues, the embryo living on its yolk; this in turn moults, and discloses the 'foetus', a transient non-feeding stage, out of which the feeding 'adolescent stage' later emerges. In Symphyla there is no known case of a pupa. A kind of 'pupa' is, however, very prevalent amongst Collembola, for the egg-membrane usually ruptures at an early stage of development, the enlarged embryo continuing its development under cover of the blastodermic cuticle.

In the character of its segmentation, the germ-band of *Pauropus* seems to resemble that of diplopods; and it is, in fact, identical with that figured by Silvestri (1933) for *Archispirostreptus gigas*. Its most noteworthy feature is the collum segment, quite devoid of appendages, and this character it shares with diplopods but with no other myriapod[1] or insect. The comparison with diplopods remains tentative, however, for there is a surprising lack of unanimity in the published accounts of the development of these myriapods. From the presence of a tritocerebral ganglion in the adult diplopod brain the presence of a pre-mandibular segment in the diplopod germ-band is to be expected, as in *Pauropus*; yet neither Heymons (1897) nor Lignau (1911) refer to it, and Pflugfelder (1932) states explicitly that it is not present. Owing to the vestigial form in which this segment commonly appears in insects, there may perhaps be some difficulty in identifying it in diplopods also, and greater weight should therefore probably be given to the statements of Silvestri (1903, 1933) and Robinson (1907) that such a segment is present in the embryos they examined (*Pachyjulus, Archispirostreptus*).

A post-maxillary collum segment, in which appendage rudiments are absent, is referred to by all recent authors on diplopod development, with one exception: Pflugfelder alone states that this segment bears a pair of second maxillae. If this is correct, it is strange that competent observers like Heymons and Silvestri can find no trace of such appendages. In *Pauropus* this segment[2] does not become part of the head; for diplopods the question is still undecided: Metchnikoff (1874) reported that two appendage-bearing segments, and two only, became associated with the head, the second pair of appendages uniting to form the lower lip (gnathochilarium). According to Heymons (1897), the

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[1] A kind of 'collum segment' is found in some adult Symphyla, but it is not the equivalent of the post-maxillary collum of Diplopoda and Pauropoda.

[2] Latzel speaks of the collum segment as the basal segment of the head; it is clear, however, that the 'cephalization' of this segment is not comparable with that of the second maxillary segment of chilopods, Symphyla, and insects.

gnathochilarium has a more complex origin, in that it arises by fusion of the first maxillae with the 'hypopharynx', i.e. sternites of the mandibular and maxillary segments; Heymons is, however, quite emphatic that the 'lamellae linguales' and 'stipites gnathochilarii' do not represent separate appendages, but arise by longitudinal division of the single pair of fused maxillae. While agreeing as to the presence of only a single pair of maxillae in the gnathochilarium, Silvestri (1903) reported that not only the maxillary sternite, but also that of the collum segment, entered into its formation, the basal sclerite (hypostome) being derived from the latter. This is confirmed by Lignau (1911); but while all other authors are agreed that at least the tergal portion of the collum segment remains separate from the head and forms the 'collum' of the larva, Lignau claims that the 'collum' develops out of the first leg-bearing segment. The remaining two publications on the development of the gnathochilarium only add to the confusion. From a very fragmentary series of *Archispirostreptus* embryos Robinson (1907) reported two maxillary segments between the mandibular and collum segments; the first pair of maxillae were stated to degenerate, while out of the second was formed the gnathochilarium. Pflugfelder (1932), on the other hand, states that the collum segment is furnished with appendages; the gnathochilarium, according to this author, arises by fusion of the first and second maxillae, the sternite of the collum segment apparently forming the 'hypostome'.

There are no adequate reasons for referring these six conflicting accounts to diversity in the forms examined. Most authors are agreed that the legless post-maxillary segment of the embryo becomes, at least in part, the collum of the adult animal; and in this respect the diplopods will then resemble *Pauropus*, but no other myriapod. There remains, however, the more debatable question of the relationship between the intermaxillary plate and maxillae of *Pauropus* and the gnathochilarium of diplopods. Does the gnathochilarium contain, in addition to the first maxillae, the equivalent of a labium? The most recent work, that of Pflugfelder, asserts that it does; on the other hand, Metchnikoff, Heymons, Silvestri, and Lignau agree that there are no second maxillae. Even if we accept Silvestri's statement that the sternite of the post-maxillary (collum) segment is incorporated into the gnathochilarium as its 'hypostome', this is by no means the equivalent of the cephalization of a labial segment and of the enlargement of the pre-oral cavity by a labium. Silvestri's work, in particular, is attested by some very clear drawings, and these are impossible to reconcile with Pflugfelder's recent work. As far as we can evaluate the conflicting accounts, it would seem that the diplopod gnathochilarium is the equivalent of the intermaxillary plate and maxillae of *Pauropus*, complicated by the incorporation of a post-maxillary sternite; and it might be regarded as an organ elaborated from some simple forerunner, of the kind found in *Pauropus*; but if Pflugfelder's work is confirmed, then the mouth-appendages of diplopods must be interpreted as a highly specialized modification of the system of appendages that form the mouth-parts in other myriapods and in insects.

The absence of a second maxillary segment in the head of *Pauropus* is of importance for the assessing of its affinities. It is clear that cephalization has not proceeded as far as in chilopods, Symphyla, and insects. In the embryology of Symphyla there is evidence of the relatively recent incorporation of this segment into the head, for it appears first as part of the abdomen rather than of the head (Tiegs, 1940), and even in insects the abdominal rather than cephalic character of its mesoderm has long been recognized (Wiesmann, 1926).

7. *Differentiation of the Mesoderm, and Formation of Somites*

By the time the germ-band has become defined, the formation of mesoderm is at an end. As already described in section 4, the mesoderm extends from just behind the stomodaeum almost to the posterior end of the germ-band. For some distance behind the stomodaeum it is heaped up several cells deep (fig. 54, Pl. 5), and even extends forward a short distance round the stomodaeum; elsewhere along the germ-band it forms a rather irregular layer, with a tendency for its cells to accumulate along the lateral margins of the germ-band in the position of the future somites.

With the pre-oral elongation of the head-lobes that takes place during the seventh day, mesodermal cells begin to spread forward from behind the stomodaeum, along the floor of the head, almost to its anterior tip. This pre-oral mesoderm thereby comes to form a continuous sheet of cells on the floor of the head (fig. 58 A, Pl. 5), but it does not spread over its lateral walls, where the process of thickening, associated with the formation of the protocerebral ganglia, is already beginning. Some cells also spread on to the stomodaeum itself (fig. 59, Pl. 5), and are the source of the mesodermal sheath of the fore-gut and eventually also of the mid-gut (see section 9). The post-oral accumulation of mesoderm is thereby much diminished. By this time the proctodaeum also has begun to form, and, like the stomodaeal ingrowth, is surrounded by a ring of mesoderm. Fig. 58 A and B, Pl. 5, will serve to show the disposition of the mesoderm in embryos at this stage of development; the drawings represent two sections from a series cut 'horizontally' through the embryo, the germ-band being therefore twice transected in a single section. Fig. 58 A shows the germ-band cut (above) through the ingrowing proctodaeum, and (below) through the head-lobe some distance in front of the stomodaeum; the median pre-oral sheet of mesoderm, and the mesoderm surrounding the base of the proctodaeum are seen in this section. In fig. 58 B the germ-band is seen transected (above) at about the level of the future third abdominal segment, and (below) a little distance behind the stomodaeum; note in this section the marked diminution of the post-oral mesoderm, but the relatively abundant mesoderm in the abdomen.

Throughout the seventh day mitoses are encountered in great numbers in the mesoderm. The tendency of its cells to aggregate into two lateral bands becomes more marked, and within these a segmentation into successive somites is becoming progressively clearer. Before the end of the seventh day most of the somites have formed (Text-fig. 5).
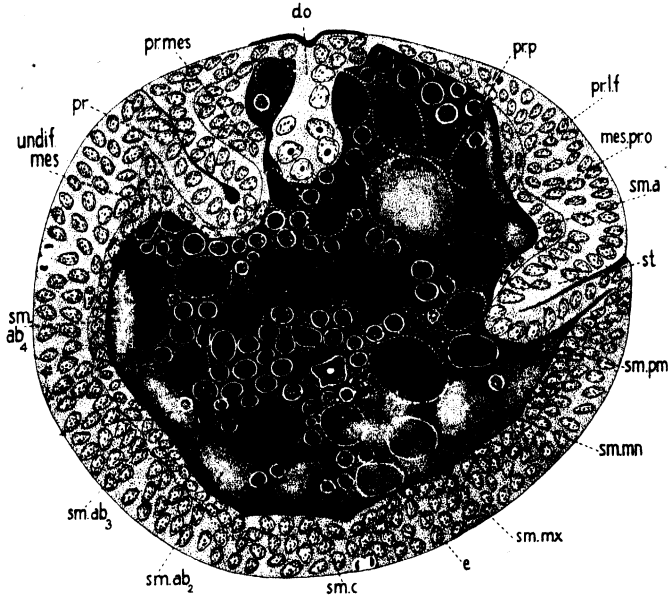
TEXT-FIG. 5. Seven-day embryo, bisected.

The embryo is at the stage in which the stomodaeal and proctodaeal ingrowths are approaching the central yolk-laden endoderm, the latter being distinguished from the rest of the 'yolk-cells' by its large nuclei and richer cytoplasmic content. The pre-antennary somites have not yet developed out of the pre-oral mesoderm, but the antennary, pre-mandibular, mandibular, maxillary, and collum somites are fully formed, and the next three abdominal somites are in course of development. Behind this the mesoderm has not yet begun to show sign of somite formation. The 'dorsal organ' is conspicuous, and its secretion has begun to form. The large posterior lobe of the protocerebral ganglion is shown, and in front of it the ectodermal thickening out of which the frontal and lateral lobes will arise. In the ventral ectoderm nerve-cord formation has hardly begun; in places the 'median mesoderm' is shown, but in longitudinally cut embryos this is usually difficult to distinguish from the underlying ectoderm.

Lettering. *d.o* 'dorsal organ'; *e* endoderm; *mes.pr.o* pre-oral mesoderm; *pr* proctodaeum; *pr.l.f* developing lateral and frontal lobes of protocerebrum; *pr.mes* proctodaeal mesoderm; *pr.p* posterior lobe of protocerebrum; *sm.a*, *sm.ab*$_2$, $_3$, $_4$, *sm.c*, *sm.mn*, *sm.mx*, *sm.pm* somites of antennary, second to fourth abdominal, collum, mandibular, maxillary, and pre-mandibular segments respectively; *st* stomodaeum; *undif.mes* mesoderm not yet differentiated into somites.

(This drawing, as well as Text-figs. 7, 8, and 9, are reconstructions from embryos cut in sagittal series. Cell-nuclei, drawn with camera lucida from the median section of the series, are used to indicate the cut surface of the bisected embryo; structures that are not cut by the median section are indicated without nuclei.)

Complete withdrawal of mesoderm from the mid-line does not take place. There survives here a narrow, and at first discontinuous, band of cells, which I shall speak of as 'median mesoderm'. Unlike the lateral bands of somites, it remains unsegmented. In some places its cells adhere closely to the adjacent ectoderm, filling out irregularities along its surface, and they may, for that

reason, often be difficult to distinguish from ectodermal cells; particularly are they apt to fill in a median groove between the two lateral thickenings of ectoderm, out of which the nerve-ganglia will form (figs. 63, 64, 65, 66, Pl. 5; fig. 67 B, Pl. 6; figs. 74, 75, 76, Pl. 6).

Within most of the segments (mandibular to fifth abdominal) there remains a small amount of mesoderm which at no time forms part of the somites. It moves into a position dorso-lateral to the somites, where it lies against the epidermis, and provides the material from which the dorso-lateral muscles develop in the late embryo. It is shown in fig. 64, Pl. 5, and is referred to more fully in section 15 (*b*) (ii).

In the procephalon there develop three pairs of somites, namely, pre-antennary, antennary, and pre-mandibular, but of these the pre-antennary are very vestigial. The mandibular, maxillary, and first four abdominal segments contain each a single pair of somites, and these are clearly distinguishable by the end of the seventh day (fig. 79, Pl. 7; Text-fig. 5). The fifth abdominal and anal segments also develop each a pair of somites, but their formation is delayed at least a day after that of the others. The presence of a distinct somite in the anal segment is evidence that the latter is a true segment and not a telson.

The fully formed somite usually presents a distinction between an outer thick somatic wall and a thinner visceral wall of flattened cells adjacent to the yolk (figs. 60, 64, Pl. 5; figs. 80, 81, Pl. 7). But even at best the visceral wall is but poorly developed, and somites are sometimes encountered where it cannot with certainty be seen. At the intersegments successive somites of the gnathal and first four abdominal segments abut on one another, and are not separated by gaps. At the height of their development many of the somites show minute cavities (fig. 81, Pl. 7), but there is no indication of those spacious coelomic vesicles that we find in other myriapods. Nor do the minute coelomic cavities of successive somites communicate at the intersegments.

A detailed account of the development and transformation of the individual somites is given in section 8.

The 'median mesoderm' gives origin to the genital tube and to certain cells, apparently neuroglial in nature, associated with the nerve-cord. For an account of these, see sections 11 and 13.

8. *Transformation of the Somites*

(i) *The Pre-antennary Somites.* These are the least developed of the somites of the procephalon, and are also the last to form within it. For some time after the succeeding two pairs of somites have already formed, the pre-oral mesoderm still lies clumped into a small median mass of cells in front of the stomodaeum (Text-fig. 5; fig. 68, Pl. 6). But during the eighth day we find, in its place, a pair of closely apposed rounded cell masses, in which there is a just recognizable distinction between visceral and somatic wall, though a coelomic cavity never develops (fig. 67 A, Pl. 6; fig. 93, Pl. 8).

During the ninth day these diminutive somites disrupt into a single clump of cells, which fills the cavity of the clypeus (fig. 103, Pl. 9). In later embryos we find these cells in process of elongation and conversion into the buccal dilator muscles (Text-fig. 7). The retractor of the clypeus (see section 15) probably also arises from this source.

These somites are evidently the equivalent of the pre-antennary somites of *Scolopendra*(Heymons, 1901), *Platyrrhacus* (Pflugfelder, 1932), and *Hanseniella* (Tiegs, 1940), and correspond also to the pre-antennary (labral) somites described for various insects—*Carausius* (Wiesmann, 1926), *Rhodnius* (Mellanby, 1936), *Locusta* (Roonwal, 1937), but in accordance with the great reduction which the somites have undergone in *Pauropus* have here become reduced to vestiges, and are not associated with any recognizable appendages. In Symphyla, where the mesoderm is very generalized, they play a role in the development of the dorsal blood-vessel, for not only do the buccal dilator muscles arise from them, as in *Pauropus*, but they also give origin to the 'funnel' of the aorta. In insects, on the other hand, the cephalic aorta arises wholly from the antennary mesoderm; in the one instance in which the further development of the pre-antennary (labral) somites has been followed, they have been found to develop into labral musculature (*Locusta*, Roonwal, 1937). In *Scolopendra* also they do not seem to aid in the formation of the dorsal vessel.

(ii) *The Antennary Somites.* These are the first somites to develop in the procephalon, and are also its largest. In embryos in which the rudiments of the antennae are just becoming perceptible in external view, the somites are seen in section, lying flattened out against the thickened epidermis of the developing appendages. They already show a clear distinction between a thin visceral and a thick somatic wall (fig. 59, Pl. 5).

With the ensuing migration of the antennae into a pre-oral position, these appendages begin to elongate and develop a cavity. Into the hollows of the antennae the somites fit. They no longer lie flattened against the epidermis, but have now assumed the form of mature somites (fig. 67 D, Pl. 6), and a little later even display each a very minute coelomic cavity (fig. 60, Pl. 5).

Transformation of the somites proceeds along very simple lines. During the ninth day they disrupt each into a loose clump of cells, which multiply and completely obliterate the cavity of the elongating appendage (fig. 110, Pl. 9); out of these will form the muscles within the antennae. Other cells spread from the bases of the antennae backwards along the roof of the head, to the side of the protocerebral ganglia, and are the source of the tergal muscles of the antennae, and also, probably, of the large dorso-lateral muscles of the head (see section 15).

The antennary mesoderm does not, as in Symphyla, contribute any cells to the formation of the stomodaeal musculature, and in accordance with the total suppression of blood-vessels it does not contain any vasoblasts. In this respect its development has evidently become much simplified, for its contribution to the formation of the vascular system in other myriapods and in

insects is very considerable. In *Scolopendra*, according to Heymons (1901), part of the cephalic aorta arises from it; in Symphyla it gives origin to the greater part of this vessel, including the antennary arteries, while in insects even the entire cephalic aorta develops from this source.

(iii) *The Pre-mandibular Somites.* In embryos in which the stomodaeum has begun to invaginate, the pre-mandibular mesoderm becomes recognizable as a pair of flattened cell-masses that lie against the thickened ectoderm a little post-orally, and in line with the row of other developing somites (fig. 61, Pl. 5). There does not seem to be any intervening 'median mesoderm'.

With the displacement of the pre-mandibular ectoderm that attends the development of the pre-oral cavity (cf. section 6 (ii) (*b*)), these masses of pre-mandibular mesoderm become drawn closer together, and now lie as a pair of rounded somites in a depression of the ectoderm immediately postero-lateral to the pre-oral cavity (Text-fig. 5; fig. 79, Pl. 7). As the pre-oral cavity becomes better defined, they come to occupy a position completely lateral to it (fig. 67 D, Pl. 6).

Meanwhile the somites have begun to elongate, and within each a small cavity appears (fig. 62, Pl. 5). During the eighth day each somite undergoes considerable elongation, growing backward into the cavity of the head to the side of the pre-oral cavity, on to the floor of the mandibular segment. The full extent of the elongating somite is to be seen in the succession of sections shown in fig. 67 C–F, Pl. 6. It is also shown in single section in fig. 71, Pl. 6, from an embryo of about the same age, in which the section was accidentally orientated to traverse most of the length of the somite; to the right the large cell-mass is the inferior wall of the pre-oral cavity, to the left is the developing mandibular ganglion and its 'ventral organ'. The prospective glandular nature of the somite is now plainly recognizable. Unlike the other somites of the procephalon, it does not disrupt, but continues to enlarge and becomes the pre-mandibular salivary gland.

The later development of the pre-mandibular gland is described in section 10 (ii).

In the embryo of *Scolopendra* (Heymons, 1901), and *Hanseniella* (Tiegs, 1940) comparatively well-developed pre-mandibular somites, furnished with coelomic cavities, are known, but they do not seem to have been recorded in any diplopod. Hoffman (1911) observed them in the embryo of the Collembolan *Tomocerus*, and even in primitive winged insects there are recognizable vestiges of them—*Xiphidium* (Wheeler, 1893), *Forficula* (Heymons, 1895), *Carausius* (Wiesmann, 1926), *Locusta* (Roonwal, 1937). But in the higher order of insects they are reduced to mere cell-aggregations. Their actual conversion into recognizable segmental organs is at present known only for *Pauropus* and *Hanseniella*, though Wheeler and Heymons had recognized the vestige of such an organ in the developing sub-oesophageal bodies and 'lymphoid tissue' of *Xiphidium* and *Scolopendra* respectively.

(iv) *The Mandibular Somites.* Like the developing somites of the antennary segment, the mandibular somites lie at first flattened out against the thickened

epidermis from which the associated appendages are beginning to form. Between them is some 'median mesoderm'. As the mandibles develop the somites take definite shape, and now show a well-formed visceral and somatic wall (fig. 79, Pl. 7). They fill the diminutive hollows of the growing mandibles, and are rather larger than most of the other somites. Within each a minute coelomic cavity appears (figs. 67 F, Pl. 6; figs. 80, 81, Pl. 7). In this condition they remain longer than the immediately adjacent maxillary and pre-mandibular somites, for these are to be seen in process of conversion into their respective glands, while the mandibular somites are still intact.

Transformation of the somites begins during the eighth day, and from them there arises nothing but the mandibular musculature. The manner of development of this musculature is bound up with the formation of the hypopharyngeal apophyses and with the peculiar character of the mandibles themselves, which in the adult *Pauropus* have each a long blade-like apodeme invaginated deeply into the head-capsule (cf. section 6 (ii) (*b*)). The formation of the apodemes begins in the 9-day embryo, by the ingrowth of the ectoderm around the lateral margins of the appendages. The form of this ingrowth will readily be visualized by reference to fig. 70, Pl. 6, and fig. 110, Pl. 9; fig. 70 has been drawn from a section that passes transversely through the floor of the maxillary segment, while fig. 110 is from a section cut 'horizontally' through the floor of the segment, i.e. from a frontal section of the head.

This ingrowth of the mandibular apodeme is already seen in the earlier embryo shown in fig. 69, Pl. 6; the outlines of the somite are still recognizable in this embryo, even though the coelomic cavity has disappeared. But in the more advanced embryos shown in fig. 70, Pl. 6, and fig. 110, Pl. 9, it has disrupted into an unorganized clump of cells, which, increasing in quantity, are in process of being drawn into the cavity of the head with the ingrowing apodeme.

The further development of the musculature of the mandible is described in section 15 (*b*) (i).

In all those myriapods that have been adequately examined on this point, a well-developed mandibular coelomic sac has been found—*Scolopendra* (Heymons, 1901), *Julus* (Heathcote, 1888), *Platyrrhacus* (Pflugfelder, 1932), *Hanseniella* (Tiegs, 1940); even in Orthoptera a large coelomic sac is present in this segment (Wheeler, 1893; Heymons, 1895; Wiesmann, 1926; Roonwal, 1937), though in higher insects it tends to disappear. In giving origin to the mandibular musculature, the somite in *Pauropus* conforms to the general scheme for other myriapods and insects; but in other respects it has evidently undergone much simplification, for in addition to the absence of any vasoblasts in its walls, it does not contribute any splanchnic mesoderm to the wall of the mid-gut or fore-gut.

(v) *The Maxillary Somites.* By the seventh day the first indication of the maxillary somites has become evident, by the accumulation of the mesoderm of the segment into two masses, which are separated by a narrow band of unsegmented median mesoderm (fig. 63, Pl. 5). During the course of the day

the segment grows in width; its ganglionic thickenings begin to form, and thereafter the developing somites move into a more lateral position in the segment, into the place where the maxillae will soon appear (fig. 64, Pl. 5). They lie, indeed, considerably more to the side than do the somites that precede them (Text-fig. 5; fig. 67 F, Pl. 7). Within each a thick somatic wall and a thin visceral wall of flattened cells are distinguishable (fig. 64, Pl. 5; fig. 79, Pl. 7). Thereafter, as the maxillae begin to form, they become more rounded, and develop each a small coelomic cavity, and therewith attain the height of their development.

Their transformation sets in unusually quickly. From the hinder end of each somite a tubular ingrowth, with just perceptible lumen, begins to grow in the direction of the yolk. These elongating somites are a characteristic feature of all embryos during the eighth day, and serve as a ready means for identifying the maxillary segment in sections (fig. 67 F, Pl. 7; fig. 80, Pl. 7).

Before long the somite becomes converted into a plainly recognizable gland rudiment. It is the maxillary (salivary) gland. It is now a narrow tube, with just perceptible lumen, and is completely doubled on itself (Text-fig. 7). At its lower end, occupying the hollow of the developing maxilla, is the remains of the somite (fig. 88, Pl. 7); this has itself become rather enlarged, mitoses are not infrequent among its cells, but the characteristic cell-disposition of the original somite is much obscured.

In parasagittal sections the separation of this clump of cells from the base of the tubular gland-rudiment is becoming apparent (fig. 81, Pl. 7). The lower end of the gland is now seen to be situated between the maxilla and the mandible, but has not yet acquired an opening to the exterior. The clump of cells that is separating from it comprises myoblasts for the formation of the maxillary musculature, and these occupy the cavity of the maxilla.

In appropriately cut sections of more advanced embryos we find the lateral margin of the maxilla growing into the cavity of the head, to form an apodeme, similar to that of the mandible, but much smaller (figs. 110, 115 B, Pl. 9). The mass of myoblasts has now separated away completely from the gland rudiment; some of them remain within the maxilla, but others become drawn farther into the cavity of the head by the ingrowing apodeme.

The further development of the salivary gland and of the muscles of the maxilla is described in sections 10 (iii) and 15 (b) (i).

In all myriapods and primitive insects that have been properly investigated, a well-formed maxillary coelomic sac has been found. In *Julus* (Heathcote, 1888) and *Hanseniella* (Tiegs, 1940), the tubular salivary gland has been found to arise from it, as in *Pauropus*. The recent work of Fahlander (1938) suggests the probable occurrence of mesodermal glands also in *Scutigera* and *Lithobius*, but embryological observations on these forms are still lacking; on the other hand, in *Scolopendra* Heymons' observations (1901) reveal the presence only of ectodermal maxillary glands, as in insects. In other respects, however, this somite in *Pauropus* seems to have undergone simplification, for it does not supply any splanchnic mesoderm to the alimentary canal.

(vi) *The Somites of the Collum Segment*. The first sign of these somites is encountered on the sixth day, when the mesoderm of the narrow collum segment becomes heaped up into two masses, with a little intervening median mesoderm (fig. 65, Pl. 5; drawn from the same embryo as fig. 63, Pl. 5).

With the development of the collum ganglion, the segment widens, and the somites move farther to the side (fig. 66, Pl. 5). Here they lie in a depression of the ectoderm, but of appendages there is no trace whatever. The mature somites are rather smaller than those of the adjacent segments, and at no time show any coelomic cavity (fig. 66, Pl. 5; figs. 79, 81, Pl. 7).

In this condition they survive well into the eighth day. Thereafter they disrupt into an unorganized clump of myoblasts which lie to the side of the ganglion (fig. 89, Pl. 7), and provide the material from which the muscles of the collum segment will form.

Although the collum segment is the equivalent, in position, of the labial segment of insects, the transformation of its somite has proceeded along remarkably simple lines; for it does not contribute any splanchnic mesoderm to the mid-gut wall, nor is there any indication of a process that might be regarded as the vestige of the formation of a segmental organ. In Collembola, on the other hand, according to Philiptschenko's work on *Isotoma* (1912), a labial salivary gland arises from it.

(vii) *The Somites of the Second, Third, and Fourth Abdominal (Three Leg-bearing) Segments*. In the region of the leg-bearing segments the germ-band is, during the seventh day, much wider than in the collum segment. The quantity of mesoderm is here also greater; it is, however, not so heaped up, but lies flattened out against the adjacent ectoderm. This is well seen in fig. 74, Pl. 7, which represents a section through the second abdominal segment, and from the same embryo as shown in figs. 63, 65, Pl. 5. Between the lateral accumulation of mesoderm is a little median mesoderm.

In rather older embryos we find the paired masses of mesoderm in process of conversion into somites (fig. 75, Pl. 7). They still lie flattened out against the ectoderm, but there is already evident an alignment of the cells which foreshadows that of the future somite.

In the section shown in fig. 67 B, Pl. 6, these flattened cell-masses are becoming rounded off, and have now assumed the form of mature somites, both visceral and somatic walls being distinguishable, though coelomic cavities have not yet appeared.

By this time recognizable ganglion rudiments are present in the abdominal segments. With the enlargement of these, the somites, as in the more anterior segments, become forced more to the sides, where they are now lodged in depressions of the ectoderm. From this ectoderm the legs are now in process of forming (fig. 76, Pl. 7).

Lodged thus in the hollows of the appendage rudiments, the abdominal somites often develop each a small coelomic cavity. In fig. 76, Pl. 7 (right side), is shown the coelomic cavity of the somite of the fourth abdominal segment of a late 8-day embryo, and in fig. 81, Pl. 7, all the somites of the

abdominal segments display such a cavity. Yet frequently the somites seem to remain permanently devoid of one, and at times even the visceral wall cannot be distinguished (fig. 76, Pl. 7, left side), its nuclei being withdrawn to the sides of the somite.

In parasagittal sections small bridges of intersegmental mesoderm may be seen between the somites (fig. 81, Pl. 7); but the minute cavities of successive somites are not continuous through these bridges.

As the limb-buds grow, the lower ends of the somites become drawn out with the elongating appendages, and therewith the regular cell-alignment of the somite wall begins to disappear (fig. 77, Pl. 7). This is the first stage in the disruption of the somites.

In rather more advanced embryos the somites break down wholly into masses of cells, which fill the hollows of the appendages, and extend from the bases of the appendages medially on to the developing ganglia (fig. 78, Pl. 7), while between successive somites the quantity of mesoderm has also begun to increase. From all this large mass of cells there arise solely myoblasts for the formation of the musculature of the abdominal segments. The development of this is described in section 15.

The most surprising feature of these somites is that they do not supply any splanchnic mesoderm to the wall of the mid-gut. This seems to be quite unique among myriapods and insects.

(viii) *The Somites of the Fifth Abdominal and Anal Segments: the Teloblastic Mesoderm.* The fifth abdominal and anal somites may conveniently be considered together. They differ from all the preceding somites in their relatively late appearance, for they do not develop until the ninth day, when most of the other somites are already in process of disruption.

Up to the eighth day, when all the more anterior somites have already appeared, the mesoderm of these segments is still an undifferentiated mass of cells that extends back from the fourth abdominal segment to the hind end of the germ-band, encircling the proctodaeum. It may be seen in Text-figs. 5 and 7, and in fig. 83, Pl. 7, the latter being a section cut transversely through the fifth segment, a little in front of the proctodaeal opening.

A similar section through a 9-day embryo (fig. 84, Pl. 7) shows that the mesoderm has spread out across the floor of the segment. Its most striking feature is now a single large primordial germ-cell, lodged at its middle, with an investment of sheath cells. This germ-cell has not hitherto been distinguishable, and it is therefore impossible to determine whether it is itself an immigrant into the mesoderm or whether it has arisen directly from the latter. At this period, also, there is to be seen a tendency for the mesoderm to develop a pair of lateral thickenings, and a section through an embryo only a little more advanced shows that these thickenings are the developing fifth abdominal somites (fig. 85, Pl. 7). In longitudinally cut embryos these somites are seen to lie immediately to the rear of the fourth abdominal somites; they are, however, considerably smaller than these, and do not display a coelomic cavity (fig. 82, Pl. 7).

After the formation of the fifth pair of abdominal somites, yet another pair develops to the rear of these, a little postero-lateral to the procto-daeum. These are even smaller than the fifth somites, and do not contain a coelomic cavity. They are the somites of the terminal (anal) segment (fig. 82, Pl. 7).

In the advanced embryo the fifth somites break down each into an irregular clump of cells (fig. 86, Pl. 7), out of which the musculature of the segment later develops (see Post-emb. Dev., sections 2, 3). The anal somites also disrupt, and may be seen in late embryos as two clumps of mesoderm cells ventro-lateral to the rectum (Text-fig. 8). It is probable that these small clumps of anal mesoderm give origin to the 'occlusor ani' muscles, two small muscles lying to the sides of the anal opening and by their con-traction pressing the lamina subanalis and lamina supranalis together (see Text-fig. 26).

After formation of the somites an unusually large amount of mesoderm remains heaped up around the single primordial germ-cell (fig. 85, Pl. 7). Only a small portion of this mesoderm is actually used in the subsequent development of the genital rudiment; the greater part of it separates away and moves into a more lateral position against the body-wall, where it is recogniz-able in the pupa as the material from which the mesoderm of the teloblastic segments will be generated in the larva. I shall refer to it as the 'teloblastic mesoderm' (fig. 87, Pl. 7); see further, Post-emb. Dev., section 3.

(ix) *General Remarks on the Somites.* In *Pauropus* the somites are remark-able among myriapods for their extreme simplicity. Bearing in mind the strongly developed coelomic sacs of other myriapods, of primitive insects, and of *Peripatus*, this simplicity must almost certainly be attributed to reduction, and is evidently correlated with the absence of a cardiac Anlage in the embryo. The absence of vestigial coelomoducts is also noteworthy, for these are present in *Hanseniella* and in many primitive insects.

The failure of the somites to contribute any splanchnic mesoderm to the intestinal wall is also remarkable, and seems, indeed, to be unique among myriapods; but this, in turn, is doubtless a simplification, and is apparently associated with the failure of the somites to spread over the mid-gut wall, where the process of heart-formation along its dorsal surface is in abeyance. That the dorsal-longitudinal muscles arise from cells that were never part of the somites (see section 7) probably has a similar explanation. The impression is, indeed, given that the whole history of the somites has been most profoundly affected in *Pauropus* by the dwarfing of the body, and its secondary effects on the vascular system.

Despite the vestigial character of the somites, it is noteworthy that they do not undergo obliteration in any segment; there is nothing comparable with the partial or even complete loss of somites that we find in the embryos of the more specialized orders of insects.

The failure of the somites to contribute to the formation of fat-body is not unexpected, for this is encountered also in Symphyla and, judging by

Heathcote's fragmentary work (1888), in the Diplopoda (*Julus*). Only in chilo-pods and insects does the fat-body arise from the somites.

9. *The Alimentary Canal and Malpighian Tubes*

(*a*) *Adult Anatomy.* The fore-gut is a simple narrow tube, which joins the mid-gut in the third abdominal segment. Its hind end is sometimes consider-ably expanded (Text-fig. 16 B), and an oesophageal valve is present, though seldom very pronounced (Text-fig. 6 D). The fore-gut is composed of a simple flat epithelium, lined throughout by chitin. Its musculature is weak, there being a layer of circular fibres, and external to these a just perceptible sheath of longitudinal fibres. The oesophageal dilator muscles are referred to in section 15.

The mid-gut is a simple sac, which extends from the third abdominal seg-ment almost into the eleventh (pre-anal) segment. Its sides and roof are com-posed of relatively very large cells, with rather deeply staining cytoplasm, and very large nuclei, and lined internally with a 'honey-comb' border. The cytoplasm of these cells is heavily charged with coarse refringent concretions (Text-fig. 6 A), and these may, at times, be present in such quantity as almost wholly to obscure the cytoplasm; sometimes, however, the concretions are completely absent, having evidently been shed in mass, and in such cases the deeply staining cytoplasm of the cells is especially apparent (Text-fig. 6 B). These concretions are not artifacts, as some writers have suspected, for they are readily seen in animals freshly teased in saline. As Schmidt (1895) cor-rectly observed, individual concretions are continually being discharged into the lumen of the mid-gut, and animals are sometimes met with in which they form a large massive bolus, eventually to be extruded through the anus (Text-fig. 6 C). There can be little doubt that the concretions are a waste product. The mid-gut epithelium is therefore the principal excretory organ in *Pauropus*, for the Malpighian tubes do not seem capable of eliminating waste sub-stances, and only seldom is there any evidence for the storage of such material in visible form in the fat-body. The concretions are evidently a product of the animal's own metabolism, for they begin to accumulate even in the embryo.

In reflected light they are white. They do not show the usual radial stria-tion of uric acid crystals. I have examined their solubility by sealing whole intestines, extracted from freshly killed animals, in depression slides ringed with vaseline (the intestine can, with practice, be drawn out in one piece by pulling upon the oesophagus). They are insoluble in water, alcohol, 4 per cent. caustic soda, and 10 per cent. acetic acid. In 10 per cent. ammonia they disappear within 2–3 hours, and in 10 per cent. hydrochloric acid within a few minutes. It is clear that they cannot be pure uric acid, though they may well be some urate. From a batch of 15 extracted intestines, from which the hind-gut with Malpighian tubes were removed, I have obtained a positive, though weak, Benedict test for uric acid. Traces of adhering fat-body could hardly be the source of the urate, for an equal number of whole animals gave
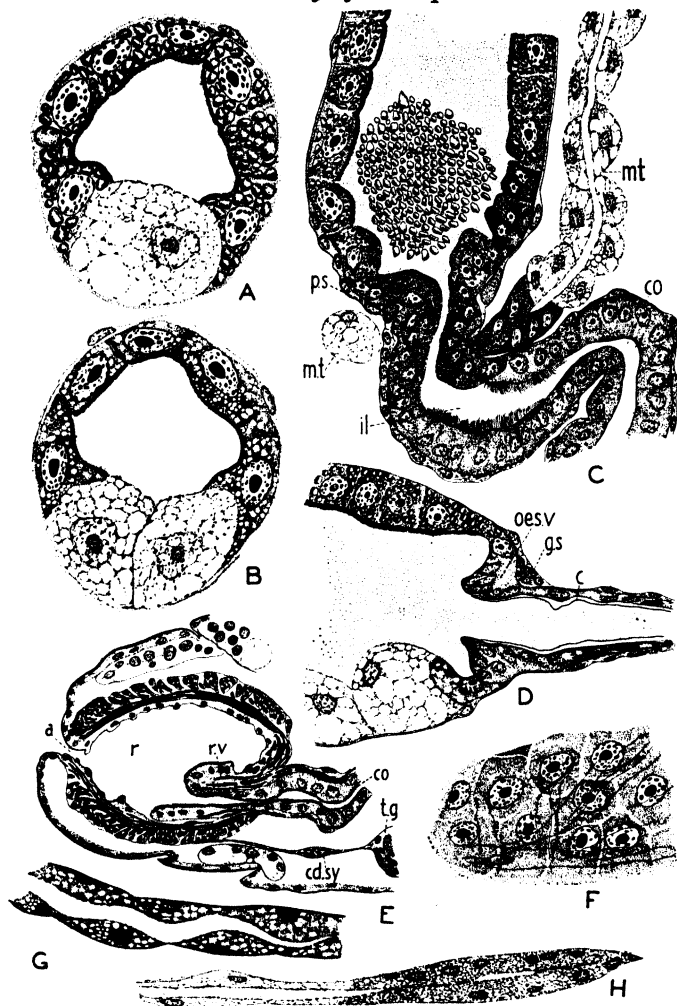
TEXT-FIG. 6. Histology of Adult Alimentary Canal.

All figures drawn to scale, except E, which is half that of the others. A. Transverse section through mid-gut, showing concretions. B. Similar section, from which the concretions have been discharged. C. Section along junction of mid-gut and hind-gut, from a 'horizontally' cut animal; within the mid-gut is a large bolus of discharged concretions; both Malpighian tubes are present in the section, one cut transversely, the other longitudinally. D. Sagittal section along junction of oesophagus and mid-gut. E. Sagittal section along hind end of abdomen, to show structure of rectum. F. Fragment of a section grazing the mid-gut wall, to show the muscle-fibrils. G. Portion of unusually well-developed Malpighian tube. H. Portion of Malpighian tube to show terminal 'glandular' portion.

Lettering. a anus; *c* chitin sheath; *cd.sy* caudal sympathetic ganglion; *co* colon; *g.s* stomachic ganglion; *il* ileum; *m.t* Malpighian tube; *oes.v* oesophageal valve; *p.s* pyloric sphincter; *r* rectum; *r.v* rectal valve; *t.g* terminal ganglion of ventral nerve-cord.

a positive test of no greater intensity. In one animal I have seen the extrusion of many concretion-laden cells from the intestinal epithelium into the lumen, and such cells are possibly the source of the animal remains occasionally found within the mid-gut.

The floor of the mid-gut is formed of cells of quite another kind. They are much larger even than the foregoing cells, but their cytoplasm is highly vacuolated and feebly staining; they are devoid of a striated border, and never display any concretions (Text-fig. 6 A, B, D). This structural differentiation of the mid-gut wall points to a separation into absorbtive and digestive zones, the latter having in addition an excretory function.

At the hindermost tip of the mid-gut the character of the epithelium changes, and its cells now resemble those of the most anterior part of the hind-gut (Text-fig. 6 c). They are devoid of concretions and of a striated border, the cell-nuclei are not exceptionally large, and the cytoplasm of the cells is heavily charged with deeply staining granules.

The mid-gut musculature is weakly developed, being, indeed, difficult to see. In sharply stained sections that graze along the outer surface of the mid-gut wall, a system of extremely fine striated fibrils can be seen; they run in all directions, and seem to be themselves devoid of nuclei (Text-fig. 6 F). As far as I have been able to observe, they are fibrillar differentiations, within a nucleated sheath of cytoplasm which encloses the entire mid-gut (Text-fig. 6 A, B). Despite their fineness, these fibrils may impart a strong churning movement to the intestinal wall, this being readily visible, on occasion, through the transparent body-wall of the animal.

A sphincter separates the mid-gut from the hind-gut. The most anterior part of the latter shows heavily granulated cells, and this points to some digestive function. Beyond this pyloric region is a short 'ileum', with strongly developed 'brush border'. Then follows a short weakly muscular 'colon', whose end is invaginated, as a 'rectal valve' into the rectum (Text-fig. 6 E). The latter is spacious, and, as usual, strongly muscular. The chitin of the hind-gut does not extend beyond the rectum.

There is a single pair of Malpighian tubes lying along the ventro-lateral surface of the mid-gut (Text-fig. 26), and extending forward almost into the third abdominal segment. They are not separately connected to the hind-gut, but are attached to a short outgrowth from the latter (Text-fig. 6 c). A lumen is always present, and it traverses the whole length of each tube; it does not, however, open into the hind-gut, but ends blindly. The microscopic structure of the Malpighian tubes presents much variation; in exceptional cases their structure may almost recall that of a normal Malpighian tube, though I have never met any that show a striated border (Text-fig. 6 G). But usually their constituent cells are heavily vacuolated, pale, and scarcely stainable, and give obvious evidence of atrophy (Text-fig. 6 C, H). The terminal part of each tube, for the length of nearly a segment, shows a different structure: its cells are smaller, the nuclei being much more closely approximated, and it presents the appearance of glandular tissue, though degenerated, and quite unlike the

rest of the Malpighian tube (Text-fig. 6 H); see further, Post-emb. Dev., section 4.

(b) Development

(i) *The Fore-gut.* Stomodaeum formation is sometimes encountered while the germ-band is still in process of differentiation out of the blastoderm, though in most embryos it is delayed till after the germ-band has formed. By the sixth day it is always present.

It arises as a small conical thickening of the ectoderm (fig. 54, Pl. 5), only a small distance from the anterior tip of the germ-band. By the parting of its cells a lumen now appears within it, its elongate slit-like orifice being readily seen in entire embryos (figs. 23, 24 B, Pl. 2). The inferior wall of the stomodaeum is markedly thinner than the superior wall (Text-fig. 5).

As the stomodaeum elongates, it acquires a loose sheath of investing mesoderm cells, which become the source, not only of the fore-gut musculature, but of that of the mid-gut as well. These cells migrate on to it from the immediately surrounding mesoderm, at the time when the somites are developing (fig. 59, Pl. 5; Text-fig. 5); the subsequent disruption of the somites that takes place during the ninth day does not, as far as I have been able to observe, yield any additional cells to the wall of the fore-gut.

During the eighth day the inner blunt end of the stomodaeum comes into contact with the clump of yolk-laden endoderm in the middle of the egg (fig. 67 D, Pl. 6). The mesodermal investment of the fore-gut has now greatly enlarged, and accumulates as a thick ridge of cells along its upper surface (Text-fig. 7).

In more advanced embryos this dorsal heaping up of mesoderm again diminishes, for many of its cells migrate on to the wall of the mid-gut. Others spread down to invest the lateral and ventral walls of the fore-gut (fig. 110, Pl. 9). This complete investment of the fore-gut by mesoderm is found chiefly towards its hinder end, and provides the cells from which the large oesophageal dilator muscles will develop. The formation of these is described in section 15 (b) (i). Only a small part of the mesoderm remains on the fore-gut itself, and out of it will develop, in the late pupa, the feeble oesophageal musculature.

In the advanced embryo the inner tip of the fore-gut is sometimes seen intruding, as an oesophageal valve, into the cavity of the mid-gut (Text-fig. 8). Even in pupae the hind end of the oesophagus is still closed (Text-fig. 9); it does not, indeed, seem to open into the mid-gut cavity till shortly before the larva emerges.

(ii) *The Hind-gut.* In most, though not all, embryos the proctodaeum arises a little later than the stomodaeum. Like the latter, it very soon develops a lumen, and acquires an investing sheath from the adjacent mesoderm (Text-fig. 5; fig. 58A, Pl. 5).

By the ninth day its blind tip has come into contact with the central yolk-laden mass of endoderm (Text-fig. 7). Hitherto it has remained a simple
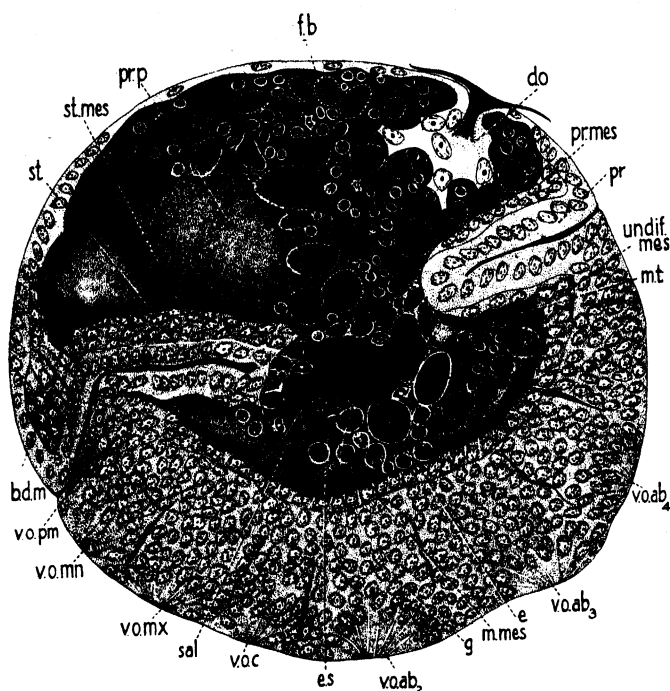
TEXT-FIG. 7. Nine-day embryo bisected (see footnote to Text-fig. 5). The section is
taken slightly to the side of the ventral mid-line, in order to show the 'ventral organs'.
The stomodaeum and proctodaeum have grown in length, and have come in con-
tact with the endoderm, which is now sharply delimited from the rest of the mass
of yolk-cells (fat-body). The somites, which by this time are disrupting, are not
visible, for they lie to the side of the row of developing nerve-ganglia; the anal and
fifth abdominal somites have not yet formed. The maxillary (salivary) gland is con-
spicuous, and shows the beginning of an 'end-sac'. The Malpighian tubes are
forming. The 'dorsal organ' is at the height of its development. 'Ventral organs'
are shown from the pre-mandibular to the fourth abdominal segment. The over-
lying ganglia are becoming larger, and successive ganglia are already partly fused.

Lettering. *b.d.m* developing buccal dilatòr muscles; *d.o* 'dorsal organ'; *e* endoderm;
e.s 'end sac' of salivary gland; *f.b* fat-body; *g* ganglionic tissue; *m.mes* median meso-
derm; *m.t* Malpighian tube; *mes.an* mesoderm of anal segment; *pr* proctodaeum;
pr.mes proctodaeal mesoderm; *pr.p* posterior lobe of protocerebrum; *sal* salivary
gland; *st* stomodaeum; *st.mes* stomodaeal mesoderm; *undif.mes* mesoderm not yet
differentiated into somites; *v.o.ab₂₋₄*, *v.o.c*, *v.o.mn*, *v.o.mx*, *v.o.pm*, 'ventral organs'
of second to fourth abdominal, collum, mandibular, maxillary, and pre-mandibular
segments respectively.

undifferentiated ingrowth, with narrow lumen, and with a single layered in-
vestment of mesodermal cells (fig. 83, Pl. 7; fig. 103, Pl. 9). But now signs of
differentiation appear within it, the lumen becoming wider, and a terminal
rectal chamber being seen in process of development. The mesodermal invest-
ment has now increased in thickness (fig. 113, Pl. 9).

In the advanced embryo the 'rectal valve' (Text-fig. 8) appears. If we compare Text-fig. 8 with fig. 113, Pl. 9, we see that the rectal valve does not arise as an ingrowth of the hinder end of the colon into the rectum, as might have been expected, but that it is a differentiation within the terminal chamber itself; for the walls of this chamber are two cell-layers in thickness, of which the inner layer, by separation, forms the valve.

Subsequently cells from the investing mesoderm spread down over the enlarging rectum, and are probably the main source of its powerful musculature. Whether additional cells are derived from the disruption of the anal somites is uncertain.

The hind-gut does not acquire an opening into the mid-gut cavity until shortly before the larva emerges.

(iii) *The Mid-gut.* The mid-gut epithelium develops chiefly out of the yolk-laden endoderm cells of the gastrula. The development of the endoderm has been described in section 2.

By the time the germ-band is forming, the endoderm cells have usually begun to acquire a richer content of cytoplasm, and therewith the yolk begins gradually to disappear. Like the partitions between the yolk-pyramids, the membrane delimiting the endoderm has now also broken down, and the cytoplasm of the latter seems to be directly continuous with that which enmeshes the remainder of the yolk (Text-fig. 5; fig. 52, Pl. 5). Not more than two nuclei are present within the endoderm; they are unusually large with prominent nucleoli, and are easily distinguishable from the nuclei of the 'yolk-cells'. They generally lie closely together, and therefore often appear in one and the same section (fig. 58 B, Pl. 5).

The cytoplasm of the endoderm cells is, at this stage, often seen to be crowded with small, rounded or rod-shaped inclusions, staining weakly with haematoxylin (fig. 54, Pl. 5). Their nature is uncertain; they do not seem to be parasitic organisms. I have never seen them in later embryos.

During the next few days the yolk continues to disappear from the endoderm cells. But the latter multiply only very slowly, and even by the eighth day not more than five or six can be counted. With increase in number they decrease in size, and come to resemble more and more closely the adjacent 'yolk-nuclei'. Their cytoplasm does not appreciably increase in quantity, and they form a loose reticulum of cells without detectable cell-walls.

During the ninth day the endoderm cells, now about ten in number, shrink from one another, and so come to enclose a spacious but irregular cavity within the middle of the yolk. This is the lumen of the future mid-gut (Text-fig. 7; figs. 103, 104 A, Pl. 9). Its limits are usually difficult to make out, for in the adjacent yolk large and irregular vacuoles are also present; and, moreover, the mid-gut cells can no longer be distinguished with certainty from yolk-cells which may lie in the neighbourhood. With this rather vaguely outlined mid-gut Anlage the tips of the stomodaeum and proctodaeum are now in contact.

TEXT-FIG. 8. Advanced embryo, bisected. (See footnote to Text-fig. 5.)

The body-wall has now been completed; note intersegmental grooves on mid-dorsal surface. The endoderm is now roofed over by splanchnic mesoderm; the endoderm cells, still highly vacuolated, are in process of forming a mid-gut, into whose cavity the fore-gut intrudes as an oesophageal valve. In the hind-gut the rectum, with intruding rectal valve, has appeared. The yolk-cells that are excluded from the mid-gut are the developing fat-body. The salivary gland extends almost to the tip of the hind-gut. The intermaxillary gland is in process of development. Of the Malpighian tube only a small part is visible, for it lies to the side of the mid-gut. The genital tube has appeared and contains a single germ-cell. In the brain the protocerebral and deutocerebral ganglia, with commissures, are seen; only a fragment of the tritocerebral ganglion is visible, for most of it lies to the side of the oesophagus. The ganglia of the ventral nerve-cord have fused, the successive ganglia projecting up to the side of the neuropilem. A fifth abdominal ganglion has separated from the teloblastic ganglion, and is showing 'ventral organ' structure; a minute anal ganglion is apparently present. In the visceral nervous system the oesophageal ganglion and the developing frontal and stomachic ganglia are recognizable.

Lettering. c.deut deutocerebral commissure; *c.t.i* inferior tritocerebral commissure; *c.prot* protocerebral commissure; *f.b* fat-body; *g.a* anal ganglion; *g.ab* $_{2, 3, 4, 5}$ ganglia of the second to fifth abdominal segments; *g.c* ganglion of collum segment; *g.fr* frontal ganglion; *g.mn* mandibular ganglion; *g.mx* maxillary ganglion; *g.oes* oesophageal ganglion; *g.pm* pre-mandibular (tritocerebral) ganglion; *g.st* stomachic ganglion; *g.t* rudiment of genital tube; *g.tbl* teloblastic ganglion; *i.mx.gl* intermaxillary gland; *m.g* mid-gut; *m.g.p* proctodaeal component of mid-gut epithelium; *mn* mandible; *m.t* Malpighian tube; *mx* maxilla; *o.a.m* developing occlusor ani muscle; *p.g.c* primordial germ cell; *r.v* rectal valve; *s* epidermal septum between right and left pre-antennary ganglia; *sal* salivary gland; *s.c* setigerous cell; *sp.mes* splanchnic mesoderm; *st.mes* stomodaeal mesoderm; *x* intersegmental groove delimiting posterior end of collum segment.

Meanwhile the mesoderm of the mid-gut has become evident. It arises in a most unusual manner, for it develops not from the somites, but from the mesoderm that lies heaped up along the dorsal wall of the stomodaeum. During the ninth day this mesoderm begins to grow backwards over the

TEXT-FIG. 9. Pupa, bisected. (See footnote to Text-fig. 5.)

The principal organs of the pupa show little advance over those depicted in Text-fig. 8. As in the latter figure, the splanchnic mesoderm has been drawn as an arching roof to the mid-gut, but to simplify the drawing no attempt has been made to indicate the lateral wall of mid-gut epithelium. Note the contrast between the very reticular endoderm cells on the floor of the mid-gut and the relatively firm endoderm cells that form its roof.

Lettering. b.a.s.o basal antennal sense organ; *c.prot* protocerebral commissure; *c.s* collum segment; *c.t.i* inferior tritocerebral commissure; *deut* deutocerebrum; *e* endoderm cells

mid-gut cells (fig. 103, Pl. 9), while at the same time it spreads down to invest them laterally; only the most ventrally situated mid-gut cells remain free from investing mesoderm. This arching roof of splanchnic mesoderm is shown in fig. 89, Pl. 7, and in Text-figs. 8 and 9.

The character of the mid-gut cells now begins to change (fig. 89, Pl. 7). They have increased to about twenty in number. Those mid-gut cells that lie beneath the investing layer of splanchnic mesoderm, and from which the roof and lateral walls of the mid-gut will develop, are now in process of forming a loose epithelium; the cytoplasm retains its feebly staining character, but is beginning to acquire a firmer texture, and it is now completely denuded of yolk. The floor of the mid-gut, on the other hand, is still constituted by large irregularly reticulate cells, within which yolk-grains are present, though in diminished numbers. Sometimes a degenerated yolk-laden cell may be present within the lumen of the mid-gut (fig. 103, Pl. 9).

In more advanced embryos the mid-gut epithelium acquires a firmer character, and therewith the distinction between floor- and roof-cells becomes very apparent (fig. 107, Pl. 9). In the roof-cells the cytoplasm is becoming darker and more dense, and the refringent concretions may already be present in considerable numbers. The floor-cells have lost their last remnant of yolk, and have merged into the general contour of the mid-gut wall. Their cytoplasm is still highly reticular and feebly staining, and they are devoid of concretions. There is no splanchnic mesoderm associated with them. In this condition the mid-gut epithelium remains until well into the pupal period (fig. 108, Pl. 9).

In the late pupa the development of the mid-gut wall is completed. The roof-cells enlarge, their nuclei acquire large nucleoli, and a striated border appears. The floor-cells, on the other hand, remain free from any trace of concretions; nor does a striated border form. The splanchnic mesoderm now completely invests the mid-gut, having spread under the floor-cells (fig. 109, Pl. 9).

The hindermost end of the mid-gut wall has an entirely different origin. In the fully developed intestine this terminal portion of the mid-gut resembles the most anterior portion of the hind-gut (Text-fig. 6 c), pointing therefore to a probable derivation from the proctodaeum of the embryo, and not from endoderm cells. This is readily confirmed when embryos after about the ninth day are examined. In fig. 113, Pl. 9, for example, may be seen the participation of the most anterior part of the proctodaeum in the formation of the mid-gut wall, the distinction between its cells, and the faintly staining

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on floor of mid-gut; *e.v* exsertile vesicle; *f.b* fat-body; *g.ab*₅ fifth abdominal ganglion; *g.fr* frontal ganglion; *g.oes* oesophageal ganglion; *g.st* stomachic ganglion; *g.t* genital tube; *g.tbl* teloblastic ganglion; *h.a* hind tip of hypopharyngeal apophysis, bending round the circum-oesophageal connective; *i.mx.gl* intermaxillary gland; *m.mes* median mesoderm (this now forms the median band of neuroglial tissue); *m.t* Malpighian tube; *mn* mandible; *mx* maxilla; *n.a* antennal nerve; *n.mx* maxillary nerve; *o.a.m* occlusor ani muscle; *oes.d.m* oesophageal dilator muscle; *prot* protocerebrum; *r.v* rectal valve; *sl* superlingua; *sp.mes* splanchnic mesoderm of mid-gut.

reticular endoderm cells, being very evident. The proctodaeal component of the mid-gut epithelium is seen also in Text-fig. 8 and fig. 114, Pl. 9. The incorporation of some proctodaeal cells into the mid-gut wall is well known for some insects, where it may have the effect of drawing even the orifices of the Malpighian tubes into the mid-gut.

(iv) *The Malpighian Tubes.* These arise, during the ninth day, as a pair of outgrowths from the anterior end of the proctodaeum, and are at first without a lumen (figs. 106, 113, Pl. 9). They grow forward on either side of the
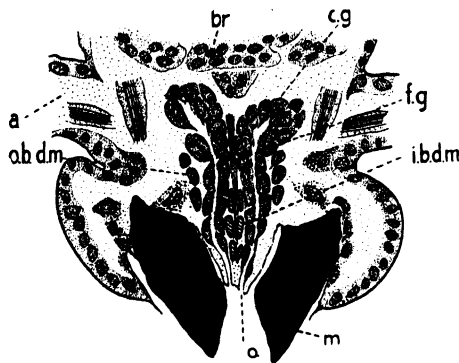
TEXT-FIG. 10. Clypeal glands. The drawing represents a frontal section of the head of a second instar larva, and shows the glands for their entire length.

Lettering. a base of antenna; *br* brain; *c.g* clypeal gland; *f.g* fragment of frontal, (visceral) ganglion: the greater part of the ganglion lies dorsal to the section; *i.b.d.m* inner buccal dilator muscle; *m* mandible; *o* orifice of clypeal gland; *o.b.d.m* outer buccal dilator muscle.

developing mid-gut, and in the late embryo extend almost the full length of the latter. By this time a lumen has appeared within them (figs. 107, 108, Pl. 9). In the relatively large size and texture of cytoplasm of their cells they present, at this time, the appearance of normal developing Malpighian tubes.

Early in the pupal period we see the first sign of the differentiation of the Malpighian tubes into a terminal part of more crowded darker cells, and a middle part in which the cells are already paler, with more widely dispersed nuclei, while at the proximal end of the tube a region with rather more crowded nuclei is seen, representing the zone of future cell-proliferation (fig. 114, Pl. 9). By the end of the pupal period the distinction has become more marked (fig. 133, Pl. 10).

The retrogression of the Malpighian tubes falls within the larval period; see Post-emb. Dev., section 5.

10. *The Glands*

(i) *The Clypeal Glands* (Text-figs. 10, 16). These glands have not hitherto been described in *Pauropus*. They lie on the floor of the clypeus, between the

inner and outer rows of buccal dilator muscles (Text-fig. 18 F). They are very simple glands, with a narrow unbranched lumen, and open, in front, on to the roof of the pre-oral cavity. Their hinder ends do not extend farther back than the bases of the antennae. Their cells are small, and without a strikingly glandular texture of cytoplasm, their secretory function being inferred from the presence of their ducts which lead into the pre-oral cavity.
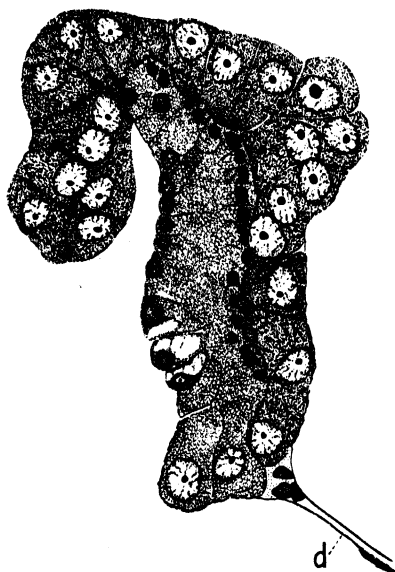
TEXT-FIG. 11. Pre-mandibular Gland; from an animal cut in sagittal section.
Lettering. d duct.

They are of ectodermal origin. In embryos aged about ten days, sections taken parallel with the floor of the clypeus display these glands as a pair of elongate columns of cells, without lumen, in process of growing backward to the side of the frontal ganglion (fig. 91, Pl. 7). The suspicion that they are ectodermal glands is at once confirmed in sections through rather less advanced embryos, where they are seen in course of development as ingrowths of cells from the roof of the pre-oral cavity (fig. 90, Pl. 7). Even in very advanced embryos a lumen is not recognizable (fig. 102, Pl. 9), and this does not seem to develop until late in the pupal period.

Both in diplopods (Reinecke, 1910) and chilopods (Fahlander, 1938), glands opening into the roof of the 'buccal cavity' have been described. They are more complex in structure than the clypeal glands of *Pauropus*. In *Scolopendra*, according to Heymons (1901), they are ectodermal ingrowths.

(ii) *The Pre-mandibular Glands* (Text-figs. 11, 16). These are a pair of relatively large glands, located dorso-laterally in the second abdominal

segment. They have already been described by Schmidt (1895) as salivary glands, and by Silvestri (1902) under the name of 'bucal gland'. Each is a compact mass of glandular tissue, and is devoid of a lumen. In addition to the large functional gland-cells, we can often distinguish, in the glands, a central band of much smaller cells, with diminutive and deeply staining nuclei. These smaller cells are presumably a reserve from which effete gland cells are replaced; for in some cases they are not present at all; and in others, as in the example shown in Text-fig. 11, apparently transitional stages are present, in which certain large gland-cells are found with nuclei of the diminutive kind. The ducts are remarkably fine tubes, with about six fusiform nuclei along their length; they closely resemble tracheae, and were indeed referred to as such by Schmidt, who did not observe their connexion with the glands. This con-nexion is with the lowest part of the gland, but they are not continued into the substance of the latter. Their orifices are in a most unexpected position, being lateral to the bases of the mandibles (Text-fig. 12). The cavity into which they drain is not the pre-oral cavity proper, but a pre-oral cavity en-larged by the inturning of the lateral margins of the clypeus (cf. section 6 (ii) (b)).

The pre-mandibular glands form out of the pre-mandibular somites. In section 8 (iii) has been given an account of the early phases of their develop-ment, up to the stage in which the somites have become converted into the recognizable rudiments of a pair of tubular glands. It now remains to describe the final phases of their development.

In late embryos, owing to the change in form which the head has undergone, the gland rudiments are best seen in frontal sections of the head, in which they may appear for their entire length in a single section. Fig. 111, Pl. 9, is a drawing of such a section, and shows the developing gland, to the side of the brain, extending deeply into the head from its point of attachment to the ectoderm just in front of the mandible. The gland is shown also in fig. 116 B, Pl. 10. It is, at this period, still a long cord of cells, but a lumen is no longer recognizable.

In the advanced embryo the position of the epidermal attachment (future orifice) of the gland undergoes displacement from a position antero-median to the mandible into a position which is actually lateral to the mandible. This will readily be seen by comparing fig. 111, Pl. 9, with fig. 119, Pl. 10, the latter figure representing a section cut approximately 'horizontally' along the head of an early pupa. The displacement seems to be associated with the development of the clypeal folds, and its character can be more readily visualized if the topography of the head of an advanced embryo, as shown, for example, in fig. 30 B, Pl. 3, be kept in mind. Much of the inferior surface of the clypeus is composed of pre-mandibular ectoderm (see section 6 (ii) (b)), and the downgrowth of the epidermis on to the side of the head will clearly have the effect of drawing the gland-attachment across the front of the base of the mandible into its position to the side of the latter.

In early pupae we see the first sign of histological differentiation of the gland, for cells with large and with small nuclei are now distinguishable (fig. 119,

Pl. 10). In later pupae the gland moves farther back into the second abdominal segment (fig. 122, Pl. 10), this backward displacement being probably due to pressure from the elongating mandibular apodeme (cf. fig. 120, Pl. 10), and perhaps also to enlargement of the brain and muscles of the head. At the
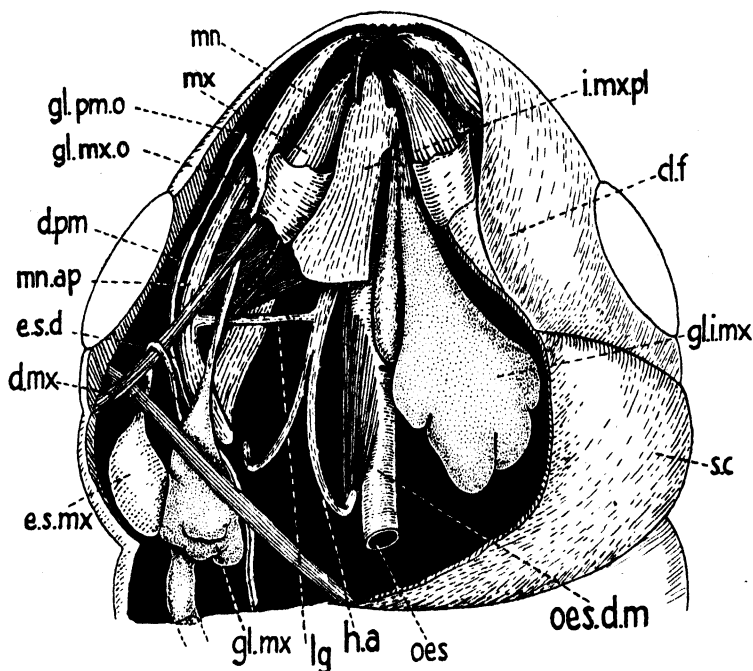
TEXT-FIG. 12. View, from below, of interior of head, to show cephalic glands and their ducts. Of the pre-mandibular gland, only the duct is shown.

Lettering. cl.f clypeal fold; *d.mx* depressor muscle of maxilla; *d.pm* duct of pre-mandibular gland; *e.s.d* duct of end-sac; *e.s.mx* end-sac of maxillary gland; *gl.i.mx* intermaxillary gland; *gl.mx* maxillary gland (only its most anterior end shown); *gl.mx.o* orifice of duct of maxillary gland; *gl.pm.o* orifice of duct of pre-mandibular gland; *h.a* hypopharyngeal apophysis; *i.mx.pl* intermaxillary plate; *lg* fibrous ligament from mandibular apodeme to hypopharyngeal apophysis; *mn* mandible; *mn.ap* mandibular apodeme; *mx* maxilla; *oes* oesophagus; *oes.d.m* oesophageal dilator muscle; *s.c* collum segment.

same time the hitherto elongate form of the gland is lost, and it assumes the compact clumped condition of the definitive organ.

The duct of the gland is now also in course of formation. It seems to be of epidermal and not mesodermal origin; but critical observations on this point are difficult to make. In early pupae the epidermal attachment of the gland is in the angle between the mandible and the clypeal fold (fig. 119, Pl. 10), and it lies immediately below the elongating mandibular apodeme, which is therefore not present in the same 'horizontally' cut section. When, in rather later

pupae, the inner wall of the clypeal fold in the neighbourhood of the gland-attachment is examined, its cells appear markedly fusiform, with elongate nuclei (fig. 121, Pl. 10). In still later pupae, when the gland has moved back into the second abdominal segment, the duct is seen for the first time, and it is cells of this latter type out of which it is constructed. Fig. 122, Pl. 10, has been drawn to illustrate this point, and evidently suggests an epidermal rather than mesodermal origin for the duct.

Wheeler, as long ago as 1893, described in the embryo of *Xiphidium* (Orthoptera) the peculiar 'sub-oesophageal' bodies which have since been observed in many other insect embryos. Although there is still some dispute as to their origin in the embryo, weight of evidence favours their derivation from the pre-mandibular mesoderm. In the embryo of *Scolopendra* there is found, in their place, some transient 'lymphoid tissue' (Heymons, 1901). Wheeler was the first to suggest a derivation of the sub-oesophageal bodies of insects from an excretory gland, and was led to compare them with the 'green gland' of Malacostraca. Heymons expressed a similar idea: 'The possibility may perhaps not be wholly excluded, that in it (lymphoid tissue) we have the modified remains of a kind of nephridium or primitive cephalic excretory gland.' These predictions have been fulfilled by the discovery, in the embryo of Symphyla, of a large pre-mandibular segmental organ, in which the end-sac assumes the form of a tubular nephrocytic organ (Tiegs, 1940); but here the gland disrupts shortly after the larva leaves the egg, and only the associated nephrocytic organ survives. In *Pauropus*, on the other hand, the gland remains even in the adult animal. It is surprising, however, to find that here its tubular character is completely obscured as development proceeds.

Wheeler, who first described the sub-oesophageal bodies in insect embryos, does not seem to have recognized the resemblance of their cells to nephrocytes, though his drawings plainly reveal it. More recent writers have compared them with the 'pericardial cells', and have attributed to them some excretory function (Heymons, 1895; Strindberg, 1916; Wiesmann, 1926; Mansour, 1927). They are generally believed to be restricted to the embryonic and early larval phases of the insect. In *Calandra oryzae*, however, they survive throughout the larval period, when they can be seen attached to the undersurface of the mycetoma, and can be recognized, though in diminished size, even in the imago (Tiegs and Murray, 1938). In this insect they undoubtedly resemble nephrocytes. To test the matter a number of larvae, pre-pupae, pupae, and adult weevils were injected by means of a micropipette with ammonia carmine, the injections being kindly made for me by Dr. F. H. Drummond. The insects were killed 24–48 hours after injection, fixed in formalin, and examined in sections. In every case the cells of the sub-oesophageal body were found to have absorbed the stain, and with an intensity in no way inferior to that of the nephrocytic cells alongside the heart.

In *Pauropus* the organ must be a salivary gland, for it is not provided with an 'end-sac', and, moreover, does not show any nephrocytic activity towards trypan blue when this dye is injected into the blood (see further, below:

maxillary gland). In *Peripatus* the segmental organ of the equivalent (third) segment is also a salivary gland, though here an 'end-sac' is present. I previously referred to this gland in Symphyla as the remains of an ancestral excretory gland, since its orifice does not lie within the pre-oral cavity; but it now seems more likely that it is the remains of an ancestral salivary gland, which has survived in *Peripatus* and *Pauropus*, its retention in the latter having
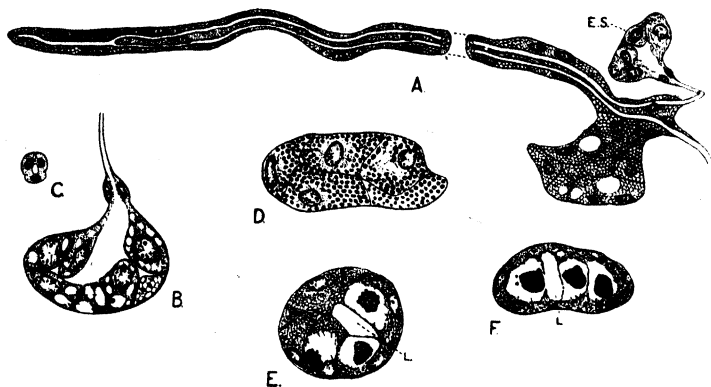
TEXT-FIG. 13. Salivary (Maxillary) Gland.

A. Optical section along gland; exit duct to right. B. Section through 'end-sac', and part of its duct. C. Transverse section through duct of 'end-sac', close to its exit from the latter. D. Section through an 'end-sac', in which the cells are unusually heavily laden with brown granules. E. Transverse section through an 'end-sac', in which the granules are aggregating into clumps. E. Final stage of aggregation of granules into compact brown clumps; to the left one of these clumps appears to be undergoing extrusion from its vacuole into the lumen of the 'end-sac'.

Lettering. e.s 'end-sac'; *l* its lumen.

perhaps been made possible by the enlargement of the pre-oral cavity due to formation of the clypeal folds.

(iii) *The (Maxillary) Salivary Glands* (Text-fig. 13). These glands, which are the largest of the salivary glands, have already been described by Silvestri (1902) under the name of 'mandibular glands'. They are a pair of long tubular glands, traversed for their complete length by a narrow channel that doubles on itself near their hinder ends, to terminate each in an 'end-sac' in the collum segment (Text-fig. 13 A). From near the anterior end of each, just below the 'end-sac', a large mass of glandular tissue depends on the floor of the collum segment. The gland-cells throughout the length of the glands are comparatively large, with finely reticular cytoplasm.

The exit ducts from the glands are a pair of narrow channels, scarcely wider than the ducts from the pre-mandibular glands, and with only sparsely distributed nuclei in their walls. They enter the head and, bending downward, pass forward median to the maxillae, to open into the pre-oral cavity just behind the bases of the mandibles (Text-fig. 12).

No description of the 'end-sacs' has hitherto been given. They are a pair of small, rounded vesicles, not more than 0·03 mm. in diameter, and are situated dorso-laterally in the collum segment, just above the large masses of salivary gland tissue that occupy much of its floor. From each 'end-sac' the narrow end-sac duct passes forward into the head, bends round the depressor muscle of the maxilla, and enters the glandular tissue (Text-figs. 12, 13 A). Shortly beyond its exit from the end-sac each duct shows a small swelling, within which three (rarely four) nuclei are lodged (Text-fig. 13 B, C). Although contractile fibrils are not visible within the cells, it seems probable that they exert a sphincter action on the end-sac.

The presence of the end-sacs first became apparent in animals that had been injected with trypan blue, as a test for the presence of nephrocytic tissue in *Pauropus*. The injections, which were made for me by Mr. A. M. Clark by the use of a Peterfi micromanipulator, were performed on animals lightly anaesthetized with ether, a small quantity of a 1 per cent. trypan blue solution being introduced through one of the posterior tergites. As might be expected with such fragile animals, the mortality was high; five animals, how- ever, survived, and on these the present account is based. Within an hour after injection of the dye the two end-sacs can be seen, through the transparent cuticle of the living animal, tinged pale blue, and thereafter the colour gradually deepens to an intense blue. Fixed preparations of animals killed 3–17 hours after injection show the dye within the substance of the end-sac. None of the other glands, nor the Malpighian tubes, are affected; nor are there any scattered nephrocytes in *Pauropus* comparable with the 'pericardial cells' of insects.

In section the end-sac is seen to be composed of relatively large uninucleate cells, with clefts from the central cavity of the vesicle extending between them (Text-fig. 13 B). The cytoplasm commonly shows a vacuolated and faintly fibrillar texture, and brownish granules may be present either in relatively small numbers (Text-fig. 13 B), or in great abundance (Text-fig. 13 D). It is upon the granules that the trypan blue is absorbed. Sometimes end-sacs are encountered in which the granules from the cytoplasm are aggregated into large clumps, which are then lodged within spacious vacuoles in the cells (Text-fig. 13 E). Within these vacuoles the granules then fuse into compact brownish masses, which are thereafter discharged into the cavity of the end- sac (Text-fig. 13 F). These are processes with which we are already familiar in the end-sacs of certain cephalic glands in Crustacea (Burian and Muth, 1924) and tracheates (Bruntz; see summary by Ehrenberg, 1924).

In the embryo the salivary glands develop out of the somites of the maxil- lary segment. The earlier phase of this development has already been de- scribed above (section 8 (v)).

The end-sacs are first seen in embryos at about the time that the proximal ends of the glands become separated from the vestiges of the original coelomic sacs, which then become resolved into myoblasts for the developing maxillae. The glands have, by this time, become completely doubled on themselves

(Text-fig. 7), the hindermost tips of the bent tubes now lying well within the collum segment, whence they extend forward to their epidermal attachment on the median aspect of the maxilla immediately to the rear of the mandibles. In each gland the rudiment of the end-sac is recognizable as a pronounced swelling at its blind tip, the cells being already distinguishable by their larger size (fig. 89, Pl. 7; fig. 117, Pl. 10).

In the advanced embryo the maxillary glands extend well back in the abdomen, where they are now seen lying to the side of the mid-gut (Text-fig. 8). In the pupa they attain their mature character, the two closely apposed limbs of the completely bent glands fusing with one another, the ducts remaining, however, apart. Their cells enlarge, and acquire a typical glandular texture. The end-sacs become withdrawn into the collum segment.

The origin of the exit ducts is difficult to determine; as far as I have been able to make out, these come from the epidermis and not from the gland-Anlage. In early pupae the anterior tip of each of the maxillary glands still lies well within the head, at the base of the maxilla, where it is connected with the epidermis on the median aspect of the maxilla just behind the mandible (fig. 123, Pl. 10). In later pupae the glandular tissue has receded into the collum segment, its connexion with the base of the maxilla being by a just perceptible string of narrow cells, whose elongate nuclei and deficient cytoplasm recall those of the epidermis at the former gland-attachment, and not of the gland-Anlage (cf. figs. 123 and 124, Pl. 10).

Maxillary glands, furnished with 'end-sacs' and mesodermal in origin, are known in Diplopoda and Symphyla. In Fahlander's recent work (1938) a maxillary gland with 'end-sac' has been described in *Scutigera* and *Lithobius*, but its presumed mesodermal origin still requires proof. In *Scolopendra*, where the development of the cephalic glands has been examined, mesodermal glands are absent (Heymons, 1901).

(iv) *The Intermaxillary Glands* (Text-figs. 12, 16 B, 17). These have already been described by Silvestri (1902) for *Allopauropus brevisetus* under the name 'maxillary gland'. In *Pauropus silvaticus* their structure is more complex than might have been expected from Silvestri's description.

They comprise a pair of large bilobed masses of glandular tissue, often closely apposed, and sometimes even fused into a single compact mass. They occupy part of the floor of the head-capsule, whence they bend down on to the floor of the collum segment. The glandular tissue consists of large irregularly vacuolated cells, with large nuclei located at the periphery. But there are also sometimes present groups of cells with smaller nuclei, from which, possibly, effete glandular cells are replaced. From the glandular tissue several irregular spacious channels pass forward, opening between the maxillae and the intermaxillary plate. The glands are presumably salivary in function, and with trypan blue give no sign of nephrocytic action. They are innervated from the sub-oesophageal ganglion by a branch of the maxillary nerve (Text-fig. 17).

The intermaxillary glands form from the ectoderm of the maxillary segment. Their development takes place in an unexpected manner, for they

arise from cells which might have been regarded as prospective ganglion cells. If, in a 10-day embryo, a section is cut transversely through the hinder part of the maxillary segment, the 'ventral organs' of that segment are found to be associated with a paired mass of cells, which is itself moulded into the contour of the hinder part of the maxillary ganglion, but is in process of separating away from the latter. This is the rudiment of the intermaxillary gland. Fig. 98, Pl. 8, in which it is shown, is drawn from an embryo in which the entire gland-Anlage, even though not completely demarcated from the ganglion, is already distinguishable from the latter by the character of its nuclei, a distinction which is, however, not always visible.

In more advanced embryos, in which the mandibular sternum has become invaginated into the pre-oral cavity, the sternum of the maxillary segment remains as the developing intermaxillary plate (cf. fig. 30 A, Pl. 3). A section directed just above the floor of this segment is shown in fig. 94 E, Pl. 8. If this is compared with fig. 98, Pl. 8, it will be seen that the maxillary 'ventral organs' have now come closely together, and form most of the tip of the inter-maxillary plate; internal to them is the developing intermaxillary gland, this being now completely separated by a cleft from the ganglion. With the change in position of the maxillary segment that has attended the enlargement of the pre-oral cavity, the intermaxillary gland-Anlage, hitherto fitting against the hinder wall of the ganglion, is brought into a position ventral to the latter (fig. 99, Pl. 8; Text-fig. 8).

Differentiation into the definitive gland now sets in. In fig. 94 E, Pl. 8, there may already be seen a tendency for the paired gland-Anlage to diverge beyond the limits of the maxillary ganglion. In more advanced embryos this divergence becomes more pronounced, while at the same time a second pair of lobes arises from each gland-Anlage (fig. 102, Pl. 9, fig. 123, Pl. 9). Within each gland the nuclei now tend to congregate around its margin, and an ill-defined lumen appears. Thereafter the simple flask-shaped character of the adult gland is attained. The cytoplasm of the cells does not assume its peculiar texture until shortly before the larva emerges from the pupa. The associated 'ventral organs' cease to be recognizable in the advanced embryo, their cells being partly incorporated into the epidermis of the intermaxillary plate and partly into the substance of the glands.

(v) *The Pseudocular Glands.* Under this name I refer to a pair of glands, by no means inconspicuous, that lie flattened out against the whole of the epithelium of the pseudoculi, lateral to the ingrown apodemes of the mandibles. They are shown in Text-figs. 16 A and 20. They are composed of large cells, with obviously glandular texture of cytoplasm, and are devoid of a duct. Their function is unknown.

The pseudocular glands develop from ectoderm. The cells from which they take origin are first distinguishable in advanced embryos, shortly after the protocerebral lobes of the developing brain have separated away from the lateral epidermis of the head. Among the normal epidermal cells certain enlarged cells are seen (figs. 111, 115 A, Pl. 9), and these soon become apparent

as the developing gland cells. Other enlarged epidermal cells, indistinguishable from those which are destined to become the gland cells, appear close by in the epidermis (fig. 111, Pl. 9); these are the setigerous cells referred to in section 14 (i).

In later embryos the prospective gland-cells separate out from the epidermis and begin to congregate between it and the tip of the ingrown mandible (fig. 100, Pl. 8). They are now much enlarged, obviously glandular in structure, and are plainly recognizable as the pseudocular gland.

It is possible that the pseudocular glands of *Pauropus* are to be compared with the 'cerebral glands' (Gehirndrüsen) of Chilopoda, to which Fahlander (1938) has recently drawn attention. Heymons (1901) long ago described the development of these glands out of the lateral epidermis of the head (*Scolopendra*) and compared them with the post-antennal organs of Tömösvary from other myriapods. Fahlander, however, points out that in the anamorphic chilopods, organs of Tömösvary and cerebral glands coexist. In chilopods these glands are innervated from the protocerebrum; in *Pauropus* I have not been able to detect a nerve supply to the pseudocular glands.

11. *The Reproductive Organs*

In the newly hatched larva the reproductive organs are still in a very rudimentary condition (Text-fig. 26 A), and consist of a narrow string of cells, without perceptible lumen, extending from the fifth abdominal segment forward into the third. The rudiment is unpaired, and lies below the intestine in a median groove along the roof of the nerve-cord. Only a single primordial germ-cell is present, and this is embedded in the genital rudiment almost at its hinder tip in the fifth segment; it is distinguishable, as usual, by its large clear nucleus. There is no discernible difference between the sexes.

The genital rudiment develops out of the unsegmented 'median mesoderm' (q.v., section 7). In the fifth abdominal segment the mesoderm remains, up to the ninth day, as a broad sheet of cells, in which the delayed formation of somites is only just beginning. Even before these somites have formed, the single primordial germ-cell is usually, though not always, recognizable in the heaped-up mesoderm, being distinguishable from the surrounding cells by its larger size and by the character of its nucleus (figs. 84, 85, Pl. 7); but whether it has arisen *in situ*, or is an immigrant from some other part of the embryo, cannot, owing to its late differentiation, be determined.

Most of the heaped-up mesoderm is used in the formation of the fifth abdominal somites and the teloblastic mesoderm (see section 8); there remains only a small aggregation of 'median mesoderm' cells, and these form a closely fitting investment for the single germ-cell (fig. 86, Pl. 7). In the advanced embryo, cells from this investing layer grow forward medially along the row of developing ganglia (Text-fig. 8), and extend eventually just into the third abdominal segment (Text-fig. 9). Owing to the presence of the single germ-cell in the fifth segment, the genital tube is here at its widest. In this condition the genital rudiment survives into the first instar larva.

The simplicity and directness of development of the genital rudiment is remarkable. In *Peripatus* (Sedgwick, 1887), and in those myriapods that have hitherto been examined—*Julus* (Heathcote, 1888), *Scolopendra* (Heymons, 1901), *Hanseniella* (Tiegs, 1940)—the genital rudiment is paired and tubular, and the lumen of the genital tube is formed by the concrescence of the cavities of successive coelomic sacs. Even in *Julus*, where the genital rudiment is an unpaired tube lying beneath the mid-gut, as in *Pauropus*, its paired origin from the two rows of coelomic sacs has been proved (Heathcote). In *Pauropus*, on the other hand, it is not even a derivative of the somites, but is developed *in situ* in the unsegmented median mesoderm. The absence of any coelomoduct associated with the genital rudiment is also noteworthy.

In the comparatively late appearance of the primordial germ-cell within the mesoderm, *Pauropus* resembles other myriapods. In many insects, on the other hand, the germ-cells are set aside as the familiar 'polar cells' at the hind end of the blastoderm, whence they migrate into the coelomic sacs when these later develop. It may well be, as Heymons (1901) has suggested, that in myriapods they undergo a similar migration, which remains, however, undetected owing to delayed differentiation of the germ-cells. The hope that *Pauropus* might throw some light on this question has not been fulfilled; the solitary germ-cell is never distinguishable except within the mesoderm of the relatively late embryo.

12. *The Haemocoele, Fat-body, and Blood*

(a) *Adult Anatomy.* In adult animals with ample reserve material in the fat-body, and with ripe gonads, the haemocoele is, for the greater part, obliterated. But when the fat-body is depleted of its reserves, and when the reproductive organs are immature, quite a spacious haemocoele is revealed.

The fat-body is confined to the abdomen, its most anterior limit being the floor of the collum segment. When not laden with reserves, it is disposed mainly along the dorsal half of the body-cavity, there being also a thin, irregular, parietal layer which spreads down towards the bases of the legs, while there is also sometimes a thick layer beneath the nerve-cord. There is, therefore, a relatively spacious cavity to either side of the nerve-cord, these lateral neural blood-spaces (sinuses) communicating with one another by means of the epineural blood-space between the ganglionic chain and the floor of the intestine, and often also by spaces beneath the ganglia, when these are not obliterated by fat-body (fig. 109, Pl. 9). With the accumulation of reserves, the fat-body begins to encroach upon the lateral neural blood-spaces, vestiges of which are, however, usually present at the bases of the legs. The musculature of the leg-bases here acts as a barrier against the bulging fat-body; sometimes, however, the fat-body succeeds in insinuating itself between the muscles, and so enters the blood-spaces of the legs, and may, on occasion, spread even as far as the tibial segment. In the head, also, there is a blood-space, but it becomes much diminished in size by the strong development of head-muscles.

In minute structure the fat-body presents nothing unusual. It is a syncytial tissue, without clear evidence of any internal cell boundaries. Its nuclei are large, with prominent nucleoli, and the protoplasm is highly vacuolated, with the usual small spherical albuminoid inclusions in the protoplasmic reticulum. On occasion the fat-body may show a fine deposit of very minute crystalline concretions; this is, however, quite unusual and there is certainly no evidence of a continued accumulation of excretory products, such as we find in Symphyla, Collembola, and some other primitive insects (*Campodea, Japyx*). The fat-body shows clear evidence of phagocytic activity. To examine for the presence of phagocytic tissue in *Pauropus*, a number of animals were injected with a minute quantity of diluted India-ink, the injection being performed through one of the hinder tergal shields. The injections were made for me by Mr. A. M. Clark with a Peterfi micromanipulator. The animals were killed from 12 to 26 hours after injection, fixed in formalin, and then cut in sections. The mere presence of ink particles within the fat-body is not evidence of phagocytosis, for with such minute and fragile animals it is impossible to avoid forcing masses of ink particles directly into the cells at the site of injection. There is, however, clear evidence in my material of incorporation of granules of ink within fat-cells quite remote from the site of injection, as, for example, within the basal segments of the legs. True phagocytosis may be recognized also by the presence of India-ink particles exclusively within the protoplasmic reticulum of the fat-cells, and not within the vacuoles, although these comprise by far the greater part of this tissue. Examples of phagocytosis are shown in Text-fig. 14.

Very rarely cells having the appearance of blood cells are met with in sections of *Pauropus*. In my India-ink material I have never seen any sign of phagocytosis by such cells.

(b) *Development*. The fat-body arises from 'yolk-cells'. The early development of these has been described in section 4.

By the time the germ-band has appeared, from 50 to 80 large nuclei, with prominent nucleoli, can be counted in the yolk. There is a scarcely perceptible condensation of cytoplasm around them, this cytoplasm forming part of the delicate syncytial reticulum that pervades the interior of the egg and supports the yolk (figs. 53, 54, 58 A, B, Pl. 5; Text-fig. 5).

As the embryo develops, the yolk gradually disappears from the supporting reticulum, which is now recognizable as the fat-body in process of formation. It fills all the space between the developing intestine and the body-wall (Text-figs. 7, 8, 9; fig. 103, 107, Pl. 9), but in the late embryo and pupa shows a tendency to shrink away from the sides of the ganglion, so forming the lateral neural channels (fig. 108, Pl. 9). Spacious lateral neural channels are, however, not a constant feature of all pupae, and in some there is even an invasion of the sub-neural spaces by fat-body. The epineural sinus arises by withdrawal of yolk from between the mid-gut and the chain of ganglia (cf. fig. 108, Pl. 9). Throughout this period the cytoplasm of the fat-body has preserved its delicate reticular character, and even in the larva shows no clearly defined internal

cell-boundaries, nor have its nuclei shown any sign of division. In the newly hatched larva the fat-body may still contain a little yolk, but this is soon absorbed (Text-fig. 26 A).

At the hinder end of the abdomen the epineural blood-space has usually become fairly well defined in the late embryo, the fat-body shrinking away
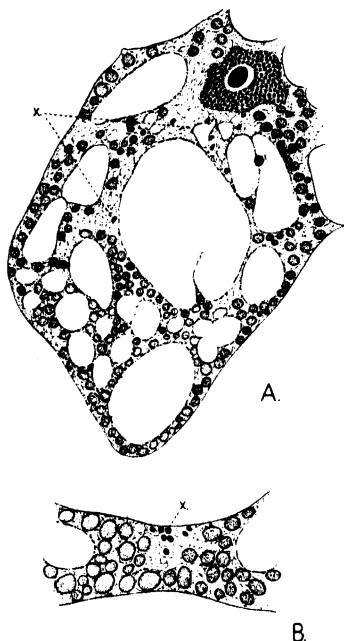
TEXT-FIG. 14. Phagocytosis by fat-body. Injected particles of India-ink shown by *X*.
A. From an animal 26 hours after injection.   B. After 12 hours.

from the space between the hind-gut and the ganglia (fig. 87, Pl. 7; fig. 106, Pl. 9).

After emergence of the larva all these blood-spaces become markedly enlarged, presumably owing to intake of water from without (cf. figs. 108 and 109, Pl. 9).

The blood-spaces of the head arise by withdrawal of the brain from the overlying epidermis (fig. 115, Pl. 9; figs. 116, 118, Pl. 10). With the enlargement of the brain and of the salivary glands during the pupal period, the fat-body is pushed back into the second abdominal segment, there being only a small amount on the floor of the collum segment (cf. Text-figs. 8 and 9).

I have not made any observations on the origin of the few problematical blood-cells.

The development of the fat-body of *Pauropus* out of the yolk-cells of the embryo is noteworthy; it is a feature that the Pauropoda share with the Symphyla, and, judging by Heathcote's fragmentary account (1888), with Diplopoda. In Chilopoda and insects, on the other hand, the entire mass of yolk becomes enclosed by mid-gut epithelium, and the fat-body develops instead from cells that are released from the walls of the somites.

13. *The Nervous System*

(a) *Structure of the Adult Nervous System.* Previous accounts of the nervous system are inadequate, and incorrect on important points. This applies especially to the brain and nerves that arise from it. The visceral nervous system has not hitherto been described.

(i) *The Ventral Nerve-cord.* This consists of a small sub-oesophageal ganglion, formed by the fusion of the mandibular and a single maxillary ganglion, the fused ganglion being withdrawn from the head into the collum segment (Text-figs. 16 B, 17); of a single collum ganglion, partly fused with the former; and of a succession of nine large ganglia, one within each of the leg-bearing segments (Text-fig. 27 A). The connectives between successive ganglia are lightly clothed with nerve-cells.

From each abdominal ganglion arise five pairs of nerves (Text-fig. 15). The most anterior of these is clothed, for some distance from its base, with nerve-cells, and passes to some of the sternal muscles of the leg. Behind it arise three other nerves, of which the hindermost supplies the ventral longitudinal and dorso-ventral muscles, while the others pass down to the muscles of the leg. To the rear of these is a fifth nerve, which runs through the abdomen on to the dorsal body-wall, probably to supply the tergal muscles; it is joined in alternate segments by a large sensory nerve from each of the great sensory setae (trichobothria).

From the sub-oesophageal ganglia arise the mandibular and maxillary nerves; it is noteworthy that the succeeding pair of nerves are the nerves of the collum segment, there being no second maxillary nerve associated with the head. These nerves are shown in Text-fig. 17. The mandibular nerve passes forward alongside the ventral longitudinal muscle, turns sharply outward, and supplies the large muscles of the mandible, and probably also the anterior end of the ventral longitudinal muscle. The maxillary nerve runs forward below the mandibular nerve, and supplies the floor of the head. It is a surprisingly large nerve, most of its fibres passing direct to the large intermaxillary gland; I have not been able to detect the small branches that presumably go to the weak maxillary muscles.

The nerves from the collum ganglion recall those of the succeeding abdominal ganglia, except that the equivalent of the nerves to the leg-muscles is lacking. There is present, also, a long thin nerve attached to the lower end of the large head-levator muscle, that arises from the floor of the collum segment (Text-fig. 17).

The terminal ganglion (Text-fig. 15) is a composite ganglion; this is at once shown by the nerves to which it gives origin, for these supply not only the last leg-bearing segment, but also those to the rear of it.
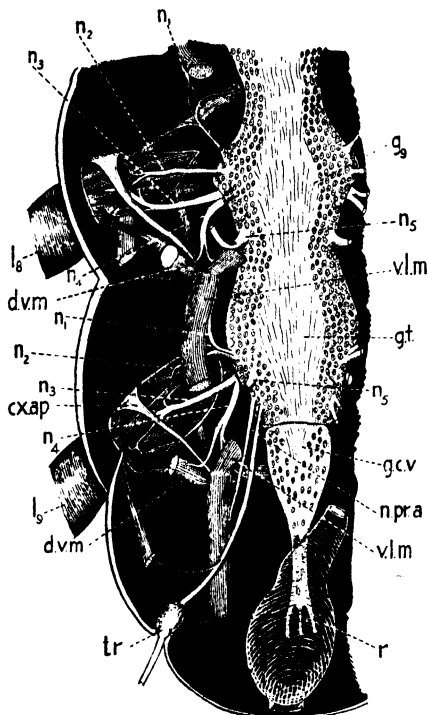
TEXT-FIG. 15. Hind end of adult animal, showing terminal portion of ganglionic chain, exposed from above. On the left is shown the distribution of the nerves. To display the nerves to the legs, it has been found necessary to omit the overlying part of the ventral longitudinal muscle.

Lettering. $cx.ap$ coxal apodeme; $d.v.m$ dorso-ventral muscle; $g.c.v$ caudal visceral ganglion; g_9 ganglion of ninth abdominal segment; $g.t$ terminal (composite) ganglion; l_8, l_9 eighth and ninth legs; n_{1-5} five pairs of segmental nerves; $n.pr.a$ nerves passing from terminal ganglion into pre-anal segment; r rectum; tr trichobothrium; $v.l.m$ ventral longitudinal muscle.

Both the nerve-cord and its segmental nerves are invested by a 'neurilemmal sheath'. This membrane is generally difficult to detect, its nuclei, which lie flattened out against the ganglia and nerves, being usually the only evidence of its presence (fig. 108, Pl. 9). But along the upper surface of the nerve-cord the 'neurilemma' is generally a thick, loosely constructed layer of spongy cells (figs. 108, 109, Pl. 9). In the neuropilem the fibres are disposed chiefly longitudinally, though, contrary to the statement by Schmidt (1895), commissural fibres are also present (they are shown in Text-fig. 16 B). Running the length of the nerve-cord there is a kind of median septum in the neuropilem,

formed of irregularly constructed cells in which the nuclei usually appear rather larger and paler than those of the ganglionic tissue. It seems to be a form of neuroglial tissue. Scattered nuclei of similar appearance are found in numbers along the boundary of the neuropilem and the ganglion cells (fig. 109, Pl. 9).

(ii) *The Brain* (Text-figs. 16, 17). This is comparatively large, its hinder end intruding far into the second abdominal segment, where it often impinges on the anterior end of the mid-gut. Seen from above it is roughly triangular, with the apex of the triangle directed forward. The protocerebrum is relatively large, and forms the hinder part of the brain. It is itself composed of three separate lobes, of which the posterior (pars intercerebralis) protrudes backwards and presents scarcely any indication of its originally paired condition. This posterior lobe forms a kind of inverted trough which arches over the oesophagus, and has its lateral margins almost in contact with the ventral nerve-cord. Anterior to it are the lateral and frontal lobes, the former widely expanded, but the latter bending downward and therefore only partially visible from above. Much of the superior and inferior surface of the protocerebrum is free from nerve-cells, the latter being distributed as a thick cortex mainly on its lateral and posterior walls. From the latter, however, the cortex encroaches from some distance on its inferior surface, and there is also a conspicuous median aggregation of nerve-cells on the dorsal surface of the brain.

The deutocerebrum, which is narrower than the protocerebrum, lies anterior to the latter; it has two lateral expansions, which extend in the direction of the pseudoculi, but otherwise presents little evidence of its originally paired condition. In a groove between the deutocerebrum and the frontal lobes of the protocerebrum lies, on each side, the ascending arm of the hypopharyngeal hypophysis.

Immediately below the deutocerebrum, and even intruding a little into it, is a median septum of ganglion cells that extends down from the roof of the brain at the junction of protocerebrum and deutocerebrum. It is indicated by *g.sp* in Text-figs. 16, 17, 18 A, C. As Text-fig. 18 C shows, it is actually a paired septum, for there is a thin partition of non-ganglionic cells between its two halves. Its development shows that it is not part of the deutocerebrum at all, but that it arises from a separate ganglion-Anlage, which is itself quite distinct from the protocerebrum. The possibility that it is the vestige of the pre-antennary ganglion, i.e. ganglion of the first head-segment, is indicated; but as the evidence for this cannot be conclusive, I shall speak of it as the 'septal ganglion'.

The tritocerebral ganglion lies to the side of the oesophagus, and merges below into the sub-oesophageal ganglion (Text-fig. 17); there are, therefore, no free circumoesophageal connectives. At their upper ends the two tritocerebral ganglia unite with one another, above the oesophagus, to form the most anterior part of the brain.

The following cerebral nerves can be distinguished (Text-figs. 16, 17):
(i) A pair of relatively large nerves from the pseudoculi (*n.ps*). These curve
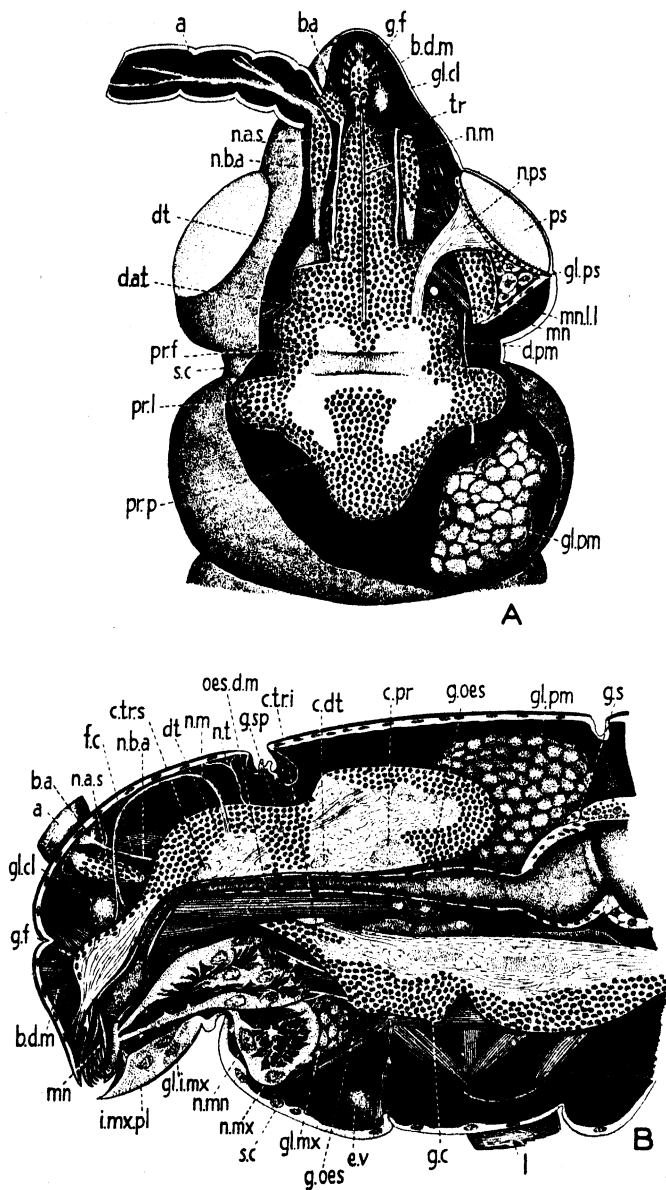
TEXT-FIG. 16. Anterior end of adult, drawn to show principally the nervous system and cephalic glands.

A. View from above. B. Bisected animal.

Lettering. *a* antenna; *b.a* basal antennal sense organ; *b.d.m* buccal dilator muscles; *c.dt* deutocerebral commissure; *c.pr* protocerebral commissure; *c.tr.i* inferior tritocerebral

back along the hinder margin of the lateral expansions of the deutocerebrum, and enter the frontal lobes of the protocerebrum.

(ii) A single very long and thin unpaired 'median nerve' (*n.m*), that originates from the roof of the deutocerebrum, and later divides into right and left branches that end in the frontal ganglion of the stomatogastric system. This nerve may perhaps be equivalent of a nerve described by Fahlander (1938) from the chilopod brain; here it arises 'at the boundary between proto- and deutocerebrum' by a pair of roots, and passes to the frontal ganglion.

(iii) A pair of thin 'tegumentary nerves', that arise from the septal ganglion, just behind the base of the 'median nerve'. It is probable that they are sensory nerves associated with the setae on the roof of the head.

(iv) A pair of large antennary nerves (*n.a.s*) that arise from the deuto-cerebrum just below the recurving nerves from the pseudoculi, and are swollen with a thick investment of nerve-cells before entering the antennae. They do not seem to supply the muscles of the antennae, and are chiefly, if not exclusively, sensory.

(v) A pair of presumably motor nerves (*n.a.m*), that arise from the deuto-cerebrum just below the foregoing. They give off several branches to the muscles at the bases of the antennae, and then enter the latter, probably to supply its muscles.

(vi) A pair of thin nerves (*n.b.a*) arising from the deutocerebrum above the antennary nerves, and supplying the basal antennary sense organ (see section 14 (iv)).

(vii) A pair of relatively large clypeal nerves (*n.cl*), arising from the sides of the tritocerebrum at the tip of the brain. They pass along the floor of the clypeus, but I have not been able to observe their termination. They are evidently the equivalent of the labral nerves of other myriapods and of insects.

(viii) A short median unpaired connective from the tritocerebrum to the frontal ganglion of the stomatogastric system. It is lightly clothed with nerve-cells, and is remarkable for the fact that it lies, as an unpaired connective, above the oesophagus.

I have been able to make only scanty and inadequate observations on the internal structure of the brain. In the protocerebrum there is no recognizable

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commissure; *c.tr.s* superior tritocerebral commissure; *d.a.t* dorsal arm of tentorium; *d.pm* duct of premandibular gland; *dt* deutocerebrum; *e.v* exsertile vesicle?; *f.c* connective between tritocerebrum and frontal ganglion; *g.c* ganglion of collum segment; *g.f* frontal ganglion; *g.oes* oesophageal ganglion; *g.s* stomachic ganglion; *g.s.oes* sub-oesophageal ganglion; *g.sp* 'septal ganglion'; *gl.cl* clypeal gland; *gl.i.mx* intermaxillary gland; *gl.mx* maxillary gland; *gl.pm* pre-mandibular gland; *gl.ps* pseudocular gland; *i.mx.pl* intermaxillary plate; *l* first leg; *mn* mandible; *mn.l.l* lateral ligamentous connexion between mandible and head-capsule; *n.a.s* sensory nerve to antenna; *n.b.a* nerve to basal antennal sense organ; *n.m* median nerve from deutocerebrum to frontal ganglion; *n.mn* mandibular nerve; *n.mx* maxillary nerve; *n.ps* pseudocular nerve; *n.t* tegumentary nerve; *oes.d.m* oesophageal dilator muscle; *pr.f* frontal lobe of protocerebrum; *pr.l* lateral lobe of protocerebrum; *pr.p* posterior lobe of protocerebrum; *ps* pseudoculus; *s.c* collum segment; *tr* tritocerebrum.

differentiation of globuli cells, in which respect the brain of *Pauropus* recalls that of Symphyla (Holmgren, 1916) and of *Geophilus* (Fahlander, 1938). Traversing the neuropilem are three pairs of compact fibrous tracts, which are

Text-fig. 17. Bisected anterior end of adult *Pauropus*, drawn to show brain, sub-oeso-phageal ganglion, and collum ganglion, together with the nerves that arise from them. The frontal visceral ganglion has been omitted in order to display the clypeal nerve; the main mass of the tritocerebrum has been shown by omitting the oesophagus and oesopha-geal dilator muscles (shown in Text-fig. 16 B).

Lettering. As in Text-fig. 16.

Additional lettering. *b.dp* buccal depressor muscle; *d.f.d* descending fibres of deuto-cerebrum; *d.l.m.h* dorsal longitudinal muscle of head; *d.l.t* dorsal longitudinal tract of descending fibres; *h.a* hypopharyngeal apophysis; *l.m.h* levator muscle of head; *mn.m* musculature of mandible; *n.a.m* antennary (motor) nerve; *n.c* nerves from collum gang-lion; *n.cl* clypeal nerve; *n.t* tegumentary nerve; *oes* oesophagus; *pr* protocerebrum; *r.c* retractor muscle of clypeus; *s.r.a₁, ₂* sternal rotator muscles of antenna; *t.l.mx* tergal levator muscle of maxilla; *tr* tritocerebrum; *v.l.m* ventral longitudinal muscle; *v.l.t* ventral longitudinal tract of descending fibres.

evidently the stalks of the pedunculate bodies (p_1, p_2, p_3, Text-fig. 18 A), but in the absence of globuli cells their precise limits of origin within the cortex cannot be determined. The second and third peduncles, and probably also the first, are associated with a median mass of neuropilem, which is evidently the equivalent of the 'medial body' (Medialkörper) described by Holmgren for the brain of *Julus* and some chilopods. Three conspicuous paired masses of neuropilem, showing faint indications of glomerular structure, are present ($glom_{1, 2, 3}$), and these are perhaps the glomeruli associated with the peduncles. Immediately to the rear of the medial body is a small, transversely elongate

body of neuropilem, which has connexions with the lateral, and perhaps also frontal, lobes of the protocerebrum. It is evidently the corpus centrale. There are no ganglion cells associated with it, in which respect it resembles the corpus centrale of other myriapods and of most crustacea, but not of arachnids. Two commissures are distinguishable within the protocerebrum, viz.: (i) the principal commissure (Text-fig. 18 A, B), lying partly behind and partly below the medial body, and connecting the lateral lobes of the protocerebrum, and perhaps also deriving fibres from the pars intercerebralis, and (ii) a small commissure, which passes between the anterior glomerular masses, and apparently connects the two frontal lobes of the protocerebrum. Finally, reference must be made to three other fibre-tracts, (i) a pair of tracts connecting the neuropilem of the deutocerebrum with the lateral mass of glomerular neuropilem; (ii) a tract of fibres (*d.l.t*, Text-figs. 17, 18 B), connected with cells of the pars intercerebralis, and passing forward along the roof of the brain to enter the sub-oesophageal ganglion by way of the tritocerebrum; (iii) a tract of fibres (*v.l.t*, Text-figs. 17, 18 B), also connected with the pars intercerebralis, but passing along the floor of the brain into the deutocerebral commissure, beyond which they cannot be followed; some fibres of this tract seem to end in the neuropilem of the deutocerebrum itself. Many of the fibres considered under (2) and (3) are efferent fibres, for they can be seen to arise from cells in the pars intercerebralis. Similar fibres are shown by Holmgren in the brain of *Julus*.

The neuropilem of the deutocerebrum shows only a faint indication of glomerular structure; within this neuropilem most of the fibres of the sensory nerves from the antennae end (Text-fig. 18 A). The motor nerves to the antennae seem to be derived mainly from cells in the lateral wall of the deutocerebrum. From the roof of the deutocerebrum tracts of fibres bend down, near the midline, and, lying in front of those from the protocerebrum, pass by way of the tritocerebrum into the sub-oesophageal ganglion (*d.f.d*, Text-figs. 17, 18 A, C). The connexion of the deutocerebrum with the lateral glomerular mass of the protocerebrum has already been referred to; also the probable connexion with the pars intercerebralis. The deutocerebral commissure is very conspicuous, its component fibres curving backwards round the septal ganglion (Text-fig. 18 A).

A large part of the tritocerebral ganglion is made up of tracts of fibres that pass between the three component ganglia of the brain and the sub-oesophageal ganglion (Text-fig. 17). The clypeal nerves originate from ganglion cells in the wall of the tritocerebrum, a little anterior to the bases of the antennary nerves, i.e. in a 'pre-oral' position. Nerve-fibres from the lateral wall of the tritocerebrum can be seen passing upward into the main mass of the brain, but cannot be followed individually. The most noteworthy feature of the tritocerebrum is the presence within it of two commissures, a superior and an inferior. The latter (*c.tr.i*, Text-figs. 17, 18 C) is, as usual, sub-oesophageal, but it is not completely 'free' as in other myriapods and insects. The presence of a superior ('pre-oral') tritocerebral commissure is unexpected. Its fibres can be traced some distance down the tritocerebral ganglion, and some, at
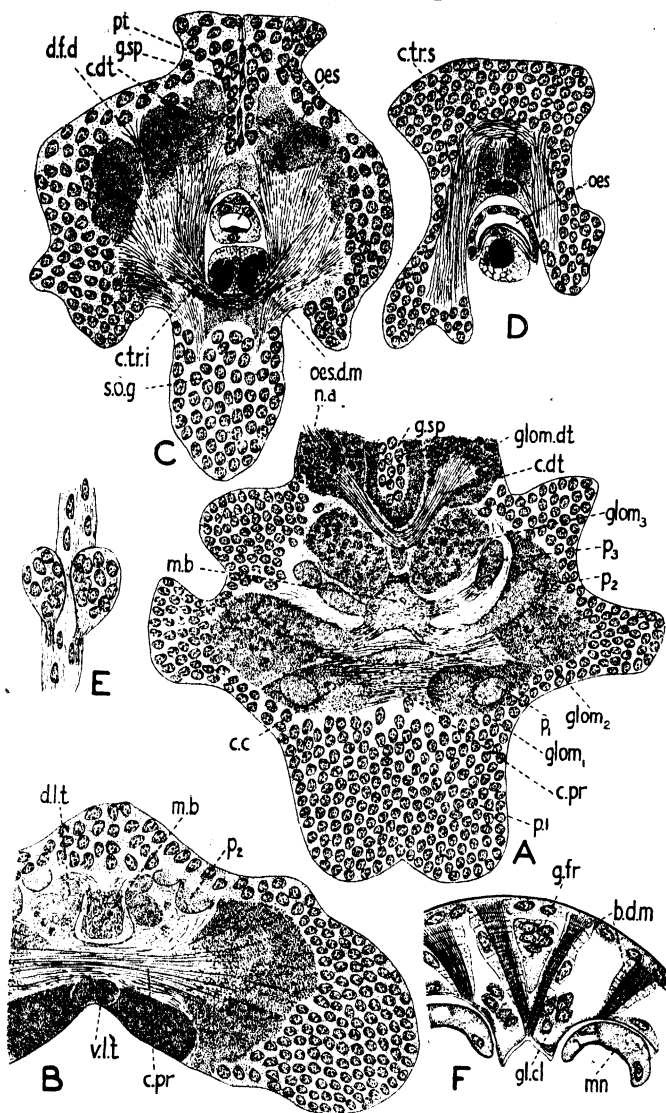
TEXT-FIG. 18. Histology of Brain and Stomatogastric Ganglia.

A. Horizontal section through protocerebrum and portion of deutocerebrum. B. Transverse section through protocerebrum, to show principal protocerebral commissure. C. Transverse section through brain to show inferior tritocerebral commissure. The section passes (above) through the deutocerebrum and 'septal ganglion' and (below) through the anterior end of the sub-oesophageal ganglion (for orientation cf. Text-fig. 17). D. Section through anterior tip of brain, showing superior tritocerebral commissure; at the sides of the oesophagus the section grazes along the anterior wall of the tritocerebrum. E. Section

least, of the crossing fibres can be seen to originate from ganglion cells in the roof of the tritocerebrum itself (Text-fig. 18 D). There seems to be no doubt as to the identification of this pre-oral tip of the brain as tritocerebrum, for the clypeal nerves and frontal connective both arise from it. The presence of a 'pre-oral' tritocerebral commissure in *Pauropus* supports the contention of Heymons (1901) that the position of the cerebral commissures in relation to the stomodaeum is determined, not by the site of origin of the ganglion in relation to the stomodaeum, but by the position into which they have moved at the time the commissures begin to develop. It may be recalled that St. Remy (1887) reported a pre-oral tritocerebral commissure in certain chilopods, though the recent work of Fahlander (1938) does not seem to support it.

(iii) *The Visceral Nervous System*. No description of this system in *Pauropus* has hitherto been given; its examination, indeed, presents considerable difficulty, and I can give only a general outline of its topography.

A stomatogastric and 'caudal' system are both present. The former seems to consist of four ganglia, as follow:

(i) A single frontal ganglion (*g.f*, Text-fig. 16 A, B), lying on the floor of the clypeus, wedged in between the inner group of buccal dilator muscles (Text-fig. 18 F). It is joined to the anterior tip of the brain (tritocerebrum) by the single short median connective above referred to. From the ganglion short nerves pass forward to the walls and floor of the clypeus. I have not been able to detect a recurrent nerve.

(ii) A pair of small oesophageal ganglia (*g.oes*, Text-fig. 16 B) that lie close together under the oesophagus, behind the posterior tips of the hypopharyngeal apophyses. They are connected by loose membrane with the immediately overlying protocerebrum and with the underlying mandibular ganglion; I cannot with certainty recognize any nerve-connexions between the oesophageal ganglia and either of these two ganglia, but it is clear that there is a connexion with nerve-fibres that can be followed a short distance along the wall of the oesophagus. In view of their remarkable development described below (they develop from cells that migrate in from the ends of the ingrown mandibles), comparison with the corpora allata of insects is at once suggested; in their microscopic structure, however, they show no evidence whatever of glandular structure, but on the contrary resemble ganglionic tissue (Text-fig. 18 E).

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along inferior wall of oesophagus, showing the oesophageal ganglia; anterior end directed downward. F. Transverse section through clypeus, to show frontal ganglion. Both groups of buccal dilator muscles are seen in the section, and between them the clypeal gland.

Lettering. b.d.m buccal dilator muscles; *c.c* corpus centrale; *c.dt* deutocerebral commissure; *c.pr* principal commissure of protocerebrum; *c.tr.i* inferior commissure of tritocerebrum; *c.tr.s* superior commissure of tritocerebrum; *d.f.d* descending fibres of deutocerebrum; *d.l.t* dorsal longitudinal fibre tracts; *dt* deutocerebrum; *g.fr* frontal ganglion; *gl.cl* clypeal gland; *glom*$_{1, 2, 3}$ first, second, and third masses of glomerular neuropilem; *glom.dt* glomerular neuropilem of deutocerebrum; *g.sp* septal (pre-antennary?) ganglion; *m.b* medial body; *mn* mandible; *n.a* sensory nerve from antenna; *oes* oesophagus; *oes.d.m* oesophageal dilator muscle; p_1, $_2$, $_3$ first, second, and third peduncles; *p.i* pars intercerebralis; *pt* partition of non-ganglionic cells in the 'septal ganglion'; *s.o.g* sub-oesophageal ganglion; *v.l.t* ventral longitudinal fibre tract.

Tentatively, therefore, we may regard them as ganglia of the stomatogastric system, despite their peculiar manner of development.

(iii) A minute stomachic ganglion (*g.s*, Text-figs. 6 D, 16 B) that lies at the posterior end of the oesophagus, at its entrance into the mid-gut.

There is no ganglion corresponding to the hypocerebral ganglion of insects. I have not been able to detect interganglionic connexions on the oesophageal wall.

The 'caudal' system of visceral nerves (Text-fig. 15) arises from the terminal ganglion of the nerve-cord. In the adult animal a relatively large clump of nerve-cells is to be seen forming the terminal (visceral) lobe of the last abdominal ganglion. From this visceral ganglion a band of nerve-fibres, sparsely clothed with nerve-cells, passes back along the floor of the terminal segment on to the inferior surface of the rectum; no other nerves arise from it. The distribution of these fibres on the intestinal wall cannot be followed.

(b) Development of the Nervous System

(i) *The Ventral Nerve-cord.* In embryos aged 6 days, and at a time when the protocerebral ganglia are already in course of formation, sections through the germ-band still reveal no sign of the development of any of the ganglia of the ventral nerve-cord. This lag in the formation of the ventral nerve-ganglia is readily seen in fig. 58 A, B, Pl. 5; the two sections are from the same embryo, fig. A showing, in the lower half of the section, the initial thickening of the head-wall, while in no other part of the section is there even an indication of the development of ganglion-Anlagen.

In rather older embryos, in which somites are in process of forming, the ectoderm along the entire post-oral length of the germ-band has become gathered into a pair of thickenings, between which only a very narrow strip of median ectoderm intervenes. This may be seen in figs. 63, 65, Pl. 5; fig. 74, Pl. 7, the three sections being taken respectively through the maxillary, collum, and second abdominal segments of a single embryo. Within the thickened bands of ectoderm the crowded nuclei lie, in places, several deep, but do not yet present any orderliness of arrangement. Between the bands of ectoderm is the unsegmented median mesoderm.

During the seventh day the developing somites begin to move into a more lateral position along the widening germ-band, and therewith the first indications of the ganglion-Anlagen become apparent (fig. 64, Pl. 5; fig. 75, Pl. 7). The latter lie to the sides of the median mesoderm and medial to the row of somites, and are directly exposed to the yolk. Lateral to them, and covered above by the somites, the ectoderm is thinner, and will become the epidermis of the appendages.[1]

In sections through rather more advanced embryos, the germ-band appears considerably thickened, and now, for the first time, the nuclei begin to display

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[1] In examining the many drawings of sections through the germ-band the reader will be struck by the apparent absence of appendage-rudiments below the somites. In whole embryos the appendages are easily seen; but in section they are scarcely recognizable because they are, at first, only gentle elevations of the epidermis, and the furrow which delimits them is, in section, hard to distinguish from natural clefts between the cells.

an orderliness of arrangement, with their long axes directed towards the surface (fig. 67 B, Pl. 6; upper half of section). Within the developing ganglia the nuclei near the surface begin to recede a little, and therewith the 'ventral organs' begin to form (fig. 76, Pl. 7); the cells immediately above them constitute the ganglion-Anlagen proper. The 'ventral organs', of which there is a single pair in each segment except the last abdominal, develop first in the more anterior segments, whence their formation spreads progressively into the segments behind. The ganglion-Anlagen of successive segments do not yet form an unbroken chain, being partially interrupted by narrow clefts at the intersegments (fig. 105, Pl. 9; Text-fig. 7).

During the ninth day the ganglia begin to take shape. They now form bulging masses on the floor of the germ-band, the clefts between successive ganglia gradually disappearing (fig. 103, Pl. 9); but the ganglion-halves still remain apart, with the median mesoderm intervening as a wedge from above. Mitoses appear in great abundance, in the cells both of the 'ventral organs' and of the ganglion-Anlagen (fig. 66, Pl. 5; figs. 86, 88, Pl. 7). The 'ventral organs' have now, also, become much more distinct. They are shown in fig. 66, Pl. 5; figs. 86, 88, Pl. 7; fig. 103, Pl. 9; Text-fig. 7.

These 'ventral organs' are, indeed, very peculiar cell-formations. Their cells are long and spindly, and tend to radiate inwards from a point on the surface. Often they display a gentle surface depression (cf. fig. 103, Pl. 9). The nuclei become withdrawn to the inner tips of the cells, in consequence of which the 'organs' become very conspicuous in sections as pale fan-shaped structures that contrast strongly with the adjacent deeply staining ganglion tissue. In order that the reader may form a better judgment of them, I have included two photographs (fig. 104 A, B, Pl. 9) of parasagittal sections along a 9-day embryo, in which four of the 'ventral organs', those of the first three abdominal segments and of the maxillary segment, can be seen.

On the ninth or tenth day the neuropilem begins to develop, and therewith the ganglion-halves become welded into a single mass. This neuropilem appears first in the more anterior segments, from where its development spreads progressively backwards. Sections through ganglia at this stage of development are shown in figs. 78, 89, Pl. 7, the former being from the second abdominal segment of a 9-day embryo, the latter from the collum segment of a 10-day embryo. The neuropilem arises as a pair of diminutive masses in the upper half of each ganglion-half, and is not roofed in by the ganglion-cells. Spreading medially, it covers in the median mesoderm, which thereby becomes included within the ganglion itself. Out of this median mesoderm will develop the median septum of neuroglia tissue, to which reference has already been made above. The ganglia are, at this stage, still associated with their 'ventral organs', and both in these and in the ganglia themselves mitosis is active. With further enlargement of the ganglia, and accumulation of neuropilem, the cleft separating the ganglion-halves becomes more and more reduced (fig. 98, Pl. 8), till eventually it vanishes (fig. 95, Pl. 8). The inclusion of the median mesoderm within the ganglia in *Pauropus* seems to be unique; in

Scolopendra also a median septum of neuroglia tissue is present, but in this case it apparently arises from the median cord (Mittelstrang) ectoderm (Heymóns, 1901).

The fate of the 'ventral organs' is not identical in all the segments. In the ganglia of the three leg-bearing segments these structures become incorporated into the ganglia themselves. Up to the ninth or tenth day a narrow median strip of ectoderm, not more than two or three cells in width, has intervened between the two rows of 'ventral organs' (fig. 78, Pl. 7). These cells now begin to spread out, covering in the 'ventral organs' from below. The latter, already considerably reduced in size, thereby become shut off from the epidermis, and are now part of the ganglia (fig. 106, Pl. 9). Their cell-orientation is soon lost, and thereafter they cannot be distinguished from the ganglion tissue.

The 'ventral organs' of the collum segment are only partially absorbed into the ganglia, their vestiges remaining in the surface ectoderm, to become converted later into the 'exsertile vesicles' of that segment (section 14 (v)). The 'ventral organs' of the maxillary and mandibular segments do not become part of the ganglia. In the maxillary segment they remain in association with the Anlage of the intermaxillary gland, and eventually become partly absorbed into that gland, and partly into the adjacent epidermis (see section 10 (iv)). Those of the mandibular segment come to occupy part of the floor of the pre-oral cavity between the mandibles (fig. 70, Pl. 6; fig. 94 D, Pl. 8); they also become separated from the ganglia, being recognizable for a short time as a thickening on the floor of the pre-oral cavity, but their cell-orientation is soon lost. They do not seem to give origin to any recognizable structure in the larva. In Symphyla the superlinguae are formed from them, but I cannot obtain certain evidence for this in *Pauropus*.

It should be observed that in *Pauropus* there is no incorporation of 'median cord' (Mittelstrang) ectoderm into the ganglia, as in Symphyla, Chilopoda, and Insecta.

Throughout the late embryonic and pupal periods both the neuropilem and the ganglionic tissue increase in quantity (figs. 107, 108, Pl. 9), and both longitudinal and commissural bundles of fibres soon become visible. Thereafter the successive ganglia become more clearly distinguishable (Text-figs. 8, 9). The sub-oesophageal ganglion forms in the late embryo by fusion of maxillary and mandibular ganglia. The collum ganglion, though less clearly demarcated than the succeeding ganglia, does not fully merge with the sub-oesophageal ganglion. An early stage in the formation of the latter ganglion is shown in fig. 99, Pl. 8.

The neurilemma seems to be derived directly from the cells of the ganglia; I have not found any evidence that the median mesoderm plays any part in its formation, though this might have been expected. Two such neurilemmal cells are shown in fig. 108, Pl. 9, where they are to be seen lying flattened out against the ganglia. The spongy neurilemmal cells on the roof of the neuropilem undoubtedly develop directly from cells of the nerve-cord. These cells may be seen in any of the following figures: figs. 95, 97, Pl. 8; figs. 107, 108,

109, Pl. 9. When first recognizable they are hardly to be distinguished from ganglion-cells (fig. 98, Pl. 8; fig. 106, Pl. 9).

Special consideration must be given to the development of the terminal ganglia, i.e. the ganglia posterior to the fourth abdominal ganglion. The development of these ganglia, like the mesoderm of these segments, is much delayed (cf. Text-fig. 7), there being no indication of them until about the ninth day. A 'ventral organ' cell-disposition then becomes apparent, and thereafter the ganglion-Anlage grows rapidly in size. A few days before the formation of the pupa, neuropilem appears in the more anterior part of this ganglionic tissue (Text-fig. 8). At the same time a constriction appears, involving both the ganglion and the 'ventral organ', which becomes separated into a more anterior part, containing the neuropilem, and a more posterior part in which neuropilem is not yet present. The more anterior of these is the developing fifth abdominal ganglion (fig. 86, Pl. 7). The hinder, however, is not, as might have been expected, the future sixth ganglion, but the locus from which the new ganglia successively develop in the growing larva. I shall speak of it as the 'teloblastic ganglion'. Its further development is described below (Post-emb. Dev., section 2).

Since there is a small somite in the anal segment, the formation of an anal ganglion is to be expected. The evidence for this is not, however, satisfactory. It seems that, in the advanced embryo, a few cells from the anal ectoderm do, in fact, become incorporated into the hinder end of the pre-anal ganglion (Text-fig. 8); but I have never seen any indication of a clearly defined anal ganglion, and there is certainly no 'ventral organ' in this terminal segment.

The most noteworthy feature of the developing nerve-cord of *Pauropus* is the presence of 'ventral organs' reminiscent of those of *Peripatus* and Symphyla. What is the significance of these peculiar structures? Kennel (1886) first described them in *Peripatus*, believing them to be the remains of a once-functional series of organs, of which a trace could still be seen in the adults of some species of *Peripatus*. The fact that, in Symphyla, their remains become converted into the exsertile vesicles seemed to support Kennel's view; and in *Pauropus* also the peculiar organs of the collum segment, themselves reminiscent of exsertile vesicles, also arise from 'ventral organs' (cf. section 10 (v)). Moreover, in the remarkable *Peripatus*-like *Xenusion*, of supposed pre-Cambrian age, each segment bore a pair of conspicuous protuberances, in the position of the 'ventral organs' (Heymons, 1928). In the embryo of *Scolopendra* the ganglia arise by the immigration of cells from invaginated pits, which themselves become incorporated into the ganglia. Heymons (1901), who described this, was led to compare them to Kennel's 'ventral organs'; but, in opposition to Kennel, he concluded that the 'ventral organs', far from being the vestiges of ancestral organs, were merely loci of cell ingrowth. But neither Kennel nor Sedgwick (1887) make any statement as to the derivation of ganglion-cells from the 'ventral organs'. In the embryo of *Hanseniella* also the ganglion-cells are mainly derived from a zone of unorientated cells between the 'ventral organs' and the ganglia, mitoses within the 'ventral organs',

being infrequent (Tiegs, 1940). In *Pauropus* mitoses are often met with in the cells of the 'ventral organs', but are in even greater abundance within the ganglia themselves; yet their eventual incorporation into some ganglia can leave no doubt that they are themselves a source of ganglion-cell formation. While this is, perhaps, not a crucial objection to Kennel's view, since it may be correlated with a reduction of exsertile vesicles in *Pauropus*, a more serious difficulty has arisen in the present work; for apparently normal 'ventral organs' appear in association with the frontal and lateral lobes of the protocerebral ganglia (see next section). These ganglia do not form part of the ventral series at all, but belong to the supra-oesophageal ganglion. Their presence in these ganglia suggests, therefore, that 'ventral organ' formation is, in some undisclosed way, bound up with the process of ganglion formation.

(ii) *The Brain*. This develops out of the following embryonic rudiments:

(i) The great protocerebral ganglia, themselves constituted out of three separate pairs of lobes, viz. the posterior, lateral, and frontal. These comprise the true supra-oesophageal ganglion, or ganglion of the acron (prostomium).

(ii) A pair of diminutive ganglia, probably to be regarded as the pre-antennary ganglia, or ganglia of the reduced first segment.

(iii) The antennary ganglia, or ganglia of the second segment, out of which the deutocerebrum will develop.

(iv) The pre-mandibular ganglia, or ganglia of the third segment, out of which will form the tritocerebrum.

There is no median unpaired ganglion corresponding to the 'archicerebrum' of Heymons. (The use of this term by Heymons has introduced some confusion into the subject. In Lankester's original form the name is applied to the ganglion of the annelid prostomium. Heymons correctly regards the entire protocerebrum as the equivalent of this ganglion, calling it, however, the 'syncerebrum', and reserving the term 'archicerebrum' for a small median unpaired component found in *Scolopendra* and *Forficula*. In Lankester's definition, however, the 'syncerebrum' connotes the ganglion of the acron, fused with a number of originally post-oral ventral ganglia.)

Almost from their first appearance the head-lobes of the embryo show a pair of pronounced lateral thickenings, the cells of which have a tendency to radiate inwards from the surface, after the manner of 'ventral organs' (fig. 58 A, Pl. 5). These thickenings are the rudiments of the posterior lobes of the protocerebral ganglia, and give the first indication of the nervous system. During the seventh day, the head-lobes having considerably enlarged, these first recognizable rudiments of the brain come to lie nearer together, on the roof of the head, from where they now begin to invaginate below the surface. Throughout the seventh day their slit-like orifices are conspicuous on the roof of the head (figs. 25, 26, Pl. 2), but during the eighth day they become closed.

Meanwhile the ectoderm of the enlarging head-lobes has markedly thickened, the zone of thickening spreading down almost to the developing pre-oral cavity. Within this thickened ectoderm a pair of new ganglionic masses may now be seen in course of formation, being located on the sides of the head,

between the invaginating posterior lobes and the stomodaeum. They are the indistinctly demarcated rudiments of the lateral and frontal lobes of the protocerebrum. These ganglion-rudiments are shown in fig. 68, Pl. 6, the section passing 'horizontally' along the head, ventral to the invaginated posterior lobes, no part of which is present in the section; cell-proliferation is in active progress, mitoses occurring mainly among the surface cells, but there is not yet evident among these any obvious orientation.

Confluent with the frontal lobes, and lying immediately to the side of the pre-oral mesoderm (future pre-antennary somites) are already to be seen the Anlagen of a pair of small ganglia. They lie just in front of the site of impending formation of the antennary ganglia, and give rise to a part of the brain quite distinct from the protocerebrum. I shall speak of them as the pre-antennary ganglia, implying a probable homology with the pre-antennary ganglia of *Scolopendra* embryos (Heymons, 1901). They soon become quite conspicuous, and are a constant feature of all embryos after about the eighth day. They give rise, moreover, to a well-defined part of the brain, viz. the 'septal ganglion' above described. I am bound to say that, as evidence for a pre-antennary segment, they are less satisfactory than might have been expected from so primitive a myriapod as *Pauropus*, for owing to crowding together of the head-ganglia they merge closely with the protocerebral lobes. They lie, moreover, lateral to the somites, and not, as might have been expected, median to them. This is probably in consequence of the remarkable displacement of segments which attends the formation of the pre-oral cavity. It should be observed that it is not the pre-antennary ganglia but the somites which are in an unusual position, for these lie close together in front of the stomodaeum.

During the eighth day all the component ganglia of the future brain have become well defined. It is hardly possible to construct a single drawing which will depict them in relation to one another; from the series shown in figs. 67 A–F, Pl. 6, however, the reader will be able to visualize them. The series represents six successive sections cut 'horizontally' through the head of an 8-day embryo. In fig. A the section passes just under the posterior lobes of the protocerebral ganglia, which are growing down from the roof of the head, only a fragment of the ganglion on the right side being visible. In the lateral and frontal lobes the more superficially placed nuclei are beginning to recede, and therewith a typical 'ventral organ' cell-disposition arises. This is also seen in the pre-antennary ganglion, which has itself now become quite distinct. In figs. B and C the large antennary ganglia are seen, lying immediately behind (below) the pre-antennary ganglia; the left ganglion already shows distinct 'ventral organ' structure. The antennae have by this time moved into a position anterior to the pre-oral cavity, the ganglia lying just medial to their bases, and therefore, unlike the more posterior ganglia, widely separated from one another. The pre-mandibular ganglia are also developing, and display a 'ventral organ'. They lie at this period immediately to the rear of the developing pre-oral cavity, only subsequently becoming invaginated into it.

They are shown in fig. F. Their post-oral position is best seen in fig. 80, Pl. 7, which is from an embryo of about the same age.

During the eighth day the peculiar radiating cell-disposition becomes more pronounced in the protocerebral ganglia, involving much of the frontal and lateral lobes (fig. 93, Pl. 8). Mitoses are in abundance throughout the enlarging ganglia. The pre-antennary ganglia have become fairly clearly demarcated from the frontal lobes. It is during this period, also, that the enlargement of the pre-oral cavity, which is proceeding, begins to draw in the 'ventral organs' of the pre-mandibular ganglia, which now come to form part of the hinder and lateral walls of this cavity. An early phase of this migration of the pre-mandibular epidermis is seen in fig. 92, Pl. 8; the drawing should be compared with figs. 67 E, F, Pl. 6, in which the ganglion is still completely post-oral in position. The pre-mandibular ganglia thereby come to lie more to the side of the pre-oral cavity, the mandibular ganglion now lying close along the hinder margin of the latter (Text-fig. 7; compare, also, fig. 80, Pl. 7, and fig. 103, Pl. 9).

During the ninth and tenth days all three lobes of the protocerebrum undergo much enlargement. The posterior lobes begin to merge with the lateral lobes, and lose connexion with the epidermis on the roof of the head, whence they originated. Their invagination cavities are now quite obliterated. Flattening out considerably, they come into almost direct contact with one another on the mid-dorsal surface of the head.

It is shortly after this, while the lateral and frontal lobes are still part of the epidermis, that the neuropilem is first seen (fig. 115 A, Pl. 9), and with its appearance the component lobes of the brain become still more firmly welded together (fig. 94, Pl. 8). The first-formed neuropilem is a transverse band passing between the lateral lobes, on the under surface and in front of the posterior lobes, and a large proportion of its axons comprise the protocerebral commissures. Thereafter the lateral and frontal lobes of the protocerebrum begin to separate away from the epidermis. This is not attended by a 'bending in' of the ganglionic masses, as is the case with the posterior lobes; instead, the adjacent epidermis closes in under them, and, together with their 'ventral organs', they lose connexion with the surface.

It is quite impossible to depict the concluding stages in the formation of the brain except by means of sections. For the purpose, reference should be made to the series of 'horizontal' sections shown in figs. 94 A, B, C, Pl. 8, to the frontal sections shown in figs. 110, 111, Pl. 9, and to the oblique section depicted in fig. 101, Pl. 8. During the tenth day the deutocerebrum is seen in process of formation. The antennary ganglia have, by this time, lost connexion with the epidermis, their 'ventral organs' becoming absorbed into them. Though the main mass of the ganglia still lies under the frontal lobes to the side of the oesophagus (fig. 110, Pl. 9), a median fusion of opposite ganglia has taken place above the oesophagus, and within this the neuropilem is already appearing (fig. 94 B, Pl. 8; fig. 111, Pl. 9; Text-fig. 8). Under this, ventro-lateral to the oesophagus, are the pre-mandibular ganglia (developing tritocerebrum) (figs. 110, 111, Pl. 9; fig. 116 A, Pl. 10): in appropriately directed sections their

'ventral organs', which are now well within the pre-oral cavity, may be seen in process of separating from the latter, thereby to become incorporated into the ganglia; at the same time the inferior tritocerebral commissure is being formed below the oesophagus (fig. 94 C, Pl. 8; fig. 116 A, Pl. 10). The pre-mandibular ganglia are now in partial continuity with both antennary and mandibular ganglia; the neuropilem between them is developing (fig. 101, Pl. 8; fig. 116 A, Pl. 10).

Special interest attaches now to the pre-antennary ganglia. With the enlargement of the frontal lobes of the protocerebrum, these ganglia are carried into an almost median position (fig. 101, Pl. 8; compare this with fig. 67, Pl. 6), but are prevented from meeting by a septum of epidermal cells that grow down from the roof of the head. This septum is shown in Text-fig. 8 and in fig. 101, Pl. 8, fig. 111, Pl. 9, and fig. 117, Pl. 10. The pre-antennary ganglia, as fig. 111 shows particularly clearly, lie between the deutocerebrum and the frontal lobes of the protocerebrum; in Text-fig. 8 the position of the epidermal septum also clearly defines their position. As fig. 101, Pl. 8, and figs. 116 A, 117, Pl. 10, show, the pre-antennary ganglia lie median to the neuropilem. There can no longer be any doubt that they are the 'septal ganglia', above referred to, in course of development. The epidermal partition between them is detectable even in the adult animal (cf. Text-fig. 18 C).

With continued growth of the ganglionic tissue and neuropilem, the brain enlarges more and more, till in the pupa its hinder end has intruded into the abdomen, just into its third segment (Text-fig. 9). It comes thereby to assume an increasingly horizontal position, the deutocerebrum lying no longer below, but actually in front of the protocerebrum, with which it is now closely merged. The pre-mandibular ganglia, hitherto completely post-oral in position, have meanwhile also begun, at their upper ends, to grow over the oesophagus (fig. 99, Pl. 8), and thereby come to form the anterior tip of the brain. The greater part of their substance remains, however, to the side of the oesophagus, so that 'free' connectives with the sub-oesophageal ganglion do not develop. The superior tritocerebral commissure is already distinguishable before the pupa forms.

(iii) *The Visceral Nervous System.* The visceral ganglia appear much later than the rest of the nervous system.

The frontal ganglion of the stomatogastric system is first seen in advanced embryos in which the neuropilem is already forming in the rest of the nervous system. It arises as a single, small, and unpaired median thickening of the roof of the oesophagus, near the hind end of the buccal dilator muscles (Text-fig. 8; fig. 99, Pl. 8); immediately behind it, to the side of the oesophagus, are the pre-mandibular (tritocerebral) ganglia. The rudiment of the ganglion soon becomes almost completely detached from the stomodaeal wall, and may now be seen wedged in between the hinder fibres of the buccal dilator muscles (fig. 91, Pl. 7). Pressing against it from behind are the upper tips of the pre-mandibular ganglia, which have now fused with one another above the oesophagus. From the point of fusion of these ganglia a short median band of

neuropilem with longitudinal axons is soon seen joining the frontal ganglion, forming its connective with the brain. Even in early pupae, however, the ganglion itself seems to remain free from neuropilem, and the latter does not become recognizable until the time that the larva emerges.

The oesophageal ganglia develop in such a surprising manner, that some doubt must be entertained as to whether they are, in truth, ganglia. They arise from the inner ends of the mandibular apodemes. In describing their development from the latter, some observations relating to the formation of the apodemes and associated parts may be included. In the 9-day embryo the ectoderm around the lateral margin of the mandibles begins to grow, as an apodeme, into the head, drawing in with it the mesoderm that is clumped at the base of the appendage (cf. section 8 (iv)). In the 10-day embryo these apodemes intrude backward in the head, as far as the superior surface of the mandibular ganglion. The developing hypopharyngeal apophyses, which have by this time begun to form (see section 14 (vi)), lie between them and the ganglion (fig. 94 D, Pl. 8). During the tenth day cells begin to separate from the inner tips of the apodemes, and grow, under the hypopharyngeal apophyses, towards one another across the upper surface of the mandibular ganglion. Fig. 115 A, Pl. 9, shows the initial phase of this process. In the rather later embryo shown in fig. 116 B, Pl. 10, there has now been formed a continuous band of cells uniting the ends of the mandibular apodemes across the mandibular ganglion; it lies immediately to the rear of the tritocerebral commissure (cf. fig. 116 A, Pl. 10). On the left side, in fig. 116 B, the section extends along a considerable length of the mandible, and shows incidentally the development of the 'lateral ligament', which will later bind the tip of the mandible to the lateral head-wall; this ligament is shown in fig. 111, Pl. 9. In the still later embryo shown in fig. 117, Pl. 10, the band of cells uniting the mandibular apodemes is beginning to develop a pair of swellings, and therewith we see the first indication of the actual ganglia; incidentally, it may be noted, a new feature has appeared in this section, namely, a connexion between the apophysis and the mandibular apodeme, and this constitutes the 'median ligament' of the latter (see section 6 (ii) (*b*)). Finally, in the advanced embryo the connecting band between the apodemes disappears, its cells being wholly concentrated in the two oesophageal ganglia (figs. 118, 119, Pl. 10). (The reader who compares fig. 118 with fig. 117 will suspect that the oesophageal ganglia of fig. 118 are surely identical with the rounded bodies labelled 'tritocerebrum' in Fig. 117. It should be explained that the hindermost tips of the tritocerebral ganglia intrude a little into the section from in front (see especially fig. 116 A, B) but are quite distinct from the oesophageal ganglia. For the relation of the latter to the tritocerebrum, see fig. 119, Pl. 10.) The ganglia, at this period, show no obvious association with the oesophagus; but in the pupa we see them clearly attached to the under surface of the latter.

The remarkable manner of formation of the oesophageal ganglia, not from the oesophageal wall, but from the bases of the mandibles, recalls current descriptions of the formation of the corpora allata of insects rather than of the

oesophageal ganglia. In *Calandra* the corpora allata come from the antennary mesoderm (Tiegs and Murray, 1938); but in all other cases investigated— *Forficula*, *Gryllus* (Heymons, 1895); *Bacillus rosii* (Heymons, 1897); *Chalicodoma* (Carrière and Bürger, 1898); *Formica* (Strindberg, 1913); *Apis* (Nelson, 1915); *Pieris* (Eastham, 1930); *Carausius* (Wiesmann, 1926; Pflugfelder, 1937) —they develop out of the ectoderm between the bases of the mandibles and maxillae. There is, however, nothing in the structure of the oesophageal ganglia of *Pauropus* to suggest any affinity with corpora allata, for they present no indication of glandular structure. In the phasmid *Carausius*, where the development of the 'pharyngeal ganglia' has been specially investigated (Wiesmann, 1927; Pflugfelder, 1937), they are found to arise from the oesophageal wall in close association with the hypocerebral ganglion. Here they appear to be of mixed glandular and nervous nature (corpora cardiaca), and cannot therefore be compared with the oesophageal ganglia of *Pauropus*.

The small stomachic ganglion develops in the usual way, by separation of cells from the hind end of the fore-gut (Text-fig. 8).

The caudal system of visceral nerves is a part of the terminal abdominal ganglion, and its development does not, therefore, present any special problem. The visceral ganglion does not, indeed, become demarcated from the terminal abdominal ganglion until late in the fourth larval stadium.

14. *The Epidermis and Some Simple Derivatives*

(i) *The Epidermis.* The differentiation of the blastoderm, during the fifth day, into provisional body-wall and germ-band, involves a gradual thinning out of the former, with attendant flattening of cells; and, in the germ-band, an increase in thickness, its closely crowded cells becoming columnar, with no very clear indication of delimiting cell-walls. In later embryos the ectoderm of the germ-band becomes even thicker, the nuclei lying often several deep (figs. 63, 65, Pl. 5); in some preparations the delimiting of cells in the ectoderm and other parts now appears quite pronounced, while in other preparations it is not perceptible. In the 'ventral organs', with their tapering and radiating cells, cell-demarcations are particularly clear (fig. 104, Pl. 9).

The great thickening of the ectoderm of the germ-band is, of course, associated with the development of the chain of nerve-ganglia and of the appendages. When the ganglia separate away from the epidermis the latter becomes reduced to a comparatively thin membrane. The epidermis of the appendages also thins out when these elongate.

During the eighth or ninth day a process of epidermal thickening begins to spread from along the margin of the germ-band over the provisional body-wall. As it proceeds, intersegmental grooves make their first appearance along the lateral walls of the embryo, their cells having a distinctly fusiform character. In the advanced embryo (fig. 29 A, Pl. 3) the thickening has spread on to the mid-dorsal surface, where the intersegmental grooves are now exceptionally deep. On the pleural walls of the abdominal segments the epidermis remains unusually thin, with widely spaced nuclei, and through it the yolk

may still be seen. It should be observed that the cells of the provisional body-wall do not degenerate before the advancing zone of epidermal thickening, but are themselves involved in the thickening, for mitoses are abundant among them.

The embryonic and pupal cuticles, to which reference has already been made in section 6 (ii), develop on about the eighth and twelfth day respectively. The definitive cuticle of the first instar larva does not form until late in the pupal period.

The great cutting setae of the first embryonic cuticle develop from large cells that appear in the epidermis during the ninth day (fig. 111, Pl. 9). These setigerous cells may still be seen in early pupae, but thereafter seem to become incorporated as ordinary cells into the epidermis, for I have not seen any in process of disruption.

The setae which adorn the body of *Pauropus* are remarkable for their variety of form. Most of them do not appear until a day before the larva emerges from the pupal sheath. The smallest setae are certainly the products of single epidermal cells, which grow beyond the surface of the epidermis and then chitinize. The extraordinary 'flagella' of the antennae, on the other hand, seem to arise by the co-operation of several cells. They begin to form in very young pupae (fig. 32 B, Pl. 3), two or three cells at the tips of the antennal rami sending out a common protoplasmic filament which thickens and then elongates, and assumes the peculiar annulated form of the flagellum. The flagella are hollow, and have a protoplasmic axis.

(ii) *The Trichobothria.* These are the great sensory setae, there being a single pair on each of the tergal scutes except the first and last (diminutive anal)—see Text-figs. 4, 23, 24. Most writers regard them as tactile sensilla; Verhoeff (1934), however, believes that as tactile organs they would be superfluous as the animal is already generously supplied with these, and suggests that they may be used for the detection of air currents. I find, however, that when a trichobothrium is gently touched with a fine needle the animal responds by turning swiftly to avoid the contact.

Each trichobothrium is a long, slender, chitinous seta, covered, for its greater length, with a very fine pubescence. At its base it has a bell-shaped expansion, which is sunk a little below the adjacent chitin. The underlying epidermis forms a thick bulbous swelling, from which the nerve passes down beside the dorso-ventral muscle into the hinder part of the ganglion of the same segment. The structure of this epidermal thickening is difficult to elucidate. Two kinds of cell are readily distinguished within it (Text-fig. 19 F, G): (i) A group of eight or nine large cells, with pale nuclei, connected by very fine filaments with the chitinous floor of the depression, within which the base of the seta is lodged (Text-fig. 19 G). It is probable that these cells are the sensory cells of the organ; I cannot, however, recognize any connexions of these cells with the almost imperceptible nerve-fibrils of the sensory nerve. (ii) Numerous small cells, with more deeply staining nuclei, some of which form a peripheral investing layer, while others form a central core of cells. From the latter a

cytoplasmic extension can be followed into the base of the seta. These smaller cells are probably not sense-cells; those which form the central core are evidently trichogen cells.
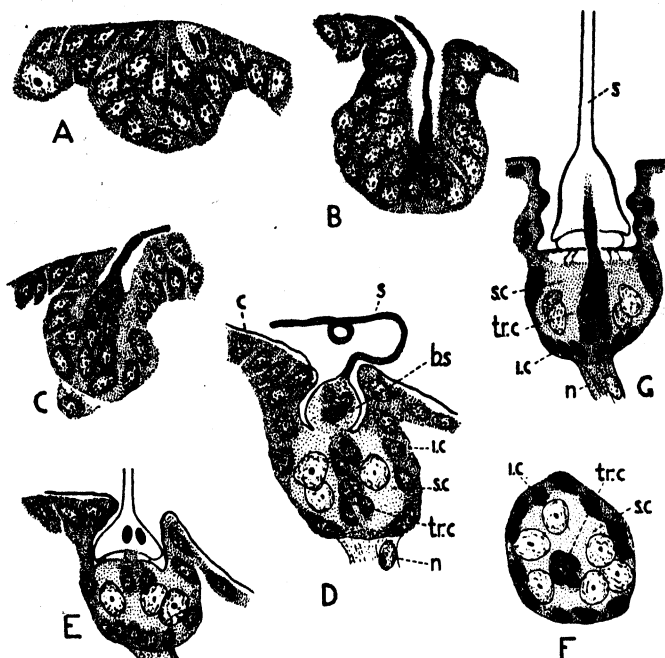
TEXT-FIG. 19.   Structure and development of the trichobothrium.
A. From late embryo. B. From an early pupa, showing initial stage in formation of seta. c. From a later pupa. D. From a very advanced pupa; chitinization has begun, and the expanded base of the seta is forming, the base being occupied by several of the core of trichogen cells. E. From a newly emerged larva; note vestiges of trichogen cells within base of seta. F, G. From an adult animal, the sections being transverse and longitudinal respectively; in G as in E only the base of the seta is indicated.

Lettering. *b.s* base of seta; *c* chitin; *i.c* investing cells; *n* nerve; *s* seta; *s.c* sense-cells; *tr.c* trichogen cells.

The rudiments of the trichobothria first become manifest on the tenth day, as deeply staining aggregations of epidermal cells in the newly forming definitive body-wall. Two pairs only are present, on the fifth and third abdominal segments respectively (figs. 29 A, 31, Pl. 3). In sections they appear as prominent thickenings of the ectoderm that intrude into the underlying yolk (fig. 94 B, Pl. 8; fig. 106, Pl. 9). Almost from the beginning the cells of the thickenings show a tendency to radiate inwards from a point on the surface (fig. 94 B, Pl. 8, left side; Text-fig. 19 A), and this is soon followed by the appearance of a cavity within the thickening. Into the cavity there intrudes a gradually lengthening filament, arising from a few rather more deeply staining cells on

its floor. From the filament will develop the seta; the deeply staining cells are trichogen cells. In this condition the developing trichobothrium is found in late embryos and early pupae (Text-fig. 19 B). In rather older pupae we see a greater development of the central core of trichogen cells, a few of which are themselves intruding into the cavity, but the sense-cells are not yet distinguishable (Text-fig. 19 C). The latter do not, indeed, become apparent till late in the pupal period, by which time the nerve has become recognizable (Text-fig. 19 D). There is now a very marked distinction between the sense-cells and the outer layer of the investing cells, while the central core of trichogen cells is also conspicuous. Of the latter, a few have clumped together to form the expanded base of the seta, on the surface of which chitin has begun to form. The seta itself has grown in length, and has coiled up beneath the pupal cuticle. In newly emerged larvae the vestiges of the cells within the expanded base of the seta are still to be seen as rounded, heavily staining clumps of degenerate chromatin (Text-fig. 20 E), but these soon disappear, leaving only a short stretch of cytoplasm from the core of the trichogen cells to enter the base of the seta.

(iii) *The Pseudoculi*. These large and problematical organs were first described by Lubbock (1868) as eyes. It is now recognized that in their structure they present little in common with visual organs, and they are usually spoken of as pseudoculi. Their general features suggest some sensory organ. Kenyon (1895) was unable to detect any nerve passing to them, but Silvestri (1902) states that they are connected by a nerve with the deutocerebrum. They present little scope for embryological investigation; their structure, however, is well worth examining.

They form a pair of very large, irregularly oval, clear, bulging protrusions of the chitin on the lateral head-wall, perfectly smooth, and free from any hairs or setae (Text-fig. 2 C). Their chitin, which is rather thin, is detached from the epidermis, which does not bulge. Silvestri attributes the glassy appearance of the pseudoculi to air-content; yet in a freshly killed animal, quickly immersed in glycerine, they do not show the familiar total reflexion of an enclosed air-pocket, the space between the epidermis and its bulging chitin being evidently occupied by fluid.

The epithelium of the pseudoculi consists of unusually large epidermal cells; it is mostly one cell in thickness, though in the mid-region they may lie several deep (Text-fig. 20 A). The nerve-fibres from the pseudoculi sweep forward towards the lateral expansion of the deutocerebrum, then curve back and enter the frontal lobe of the protocerebrum (Text-fig. 16 A). Their attachment to the pseudocular epithelium is difficult to observe; they seem to come chiefly from the inner zone of cells at its middle.

When examined in profile, there is seen, intruding into the chitin of the pseudoculus from its hinder margin, a small process of thick chitin from the head-wall (Text-fig. 2 C). Near its tip is a minute spherical globule (Text-fig. 20 B), which proves to be an excavation of the chitin from within. The protoplasm of the adjacent epidermal cells intrudes into the excavation, the

connexion being particularly well seen when the adjacent epidermis shrinks from the chitin (Text-fig. 20 c). This peculiar structure probably serves no other purpose than to prevent detachment of the chitin from its epithelium, along the hinder margin of the pseudoculus.

Of all the known types of sense-organ, the pseudoculus resembles most nearly an organ of hearing. The presence of fluid, rather than air, within it
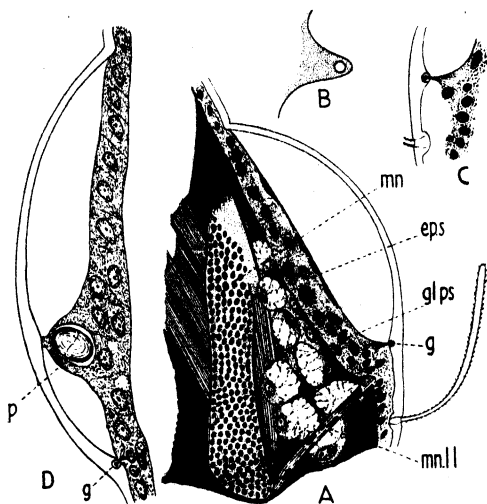
TEXT-FIG. 20. The pseudoculus and associated structures.

A. Right pseudoculus, viewed from above in optical section; inner end of mandible and pseudocular gland also shown. B. Drawing, under high magnification, of the small globule-bearing process of chitin that intrudes into the hinder margin of the chitin of the pseudoculus. C. Section through same, showing attachment of the epidermis within the globule; the attachment is sufficiently firm to resist the shrinkage which has detached the epidermis from the chitin in the region just behind (below, in drawing) the pseudoculus. D. Section along the left pseudoculus of an undescribed species of *Pauropus*, showing the 'pistil'.

Lettering. e.ps epidermis of pseudoculus; *g* globule; *gl.ps* pseudocular gland; *mn* mandible; *mn.l.l* lateral mandibular ligament; *p* 'pistil'.

would probably preclude any effective reception of air-borne sound waves, the 'globulus' of the antenna being probably the organ of hearing in air. But it should be recalled that the Pauropoda live under stones or in decaying wood, in which environment the pseudoculi could probably receive vibrations by direct contact with solid material.

In another (undescribed) larger species of *Pauropus*, sometimes found in small numbers with *P. silvaticus*, the pseudoculus conveys even more vividly the impression of a vibration-receiving organ. Traversing the cavity of the pseudoculus near its middle there is, in this species, a peculiar structure which recalls the 'pistil' described by Silvestri (1920) from *Allopauropus brevisetus*.

It is attached to the inner wall of the pseudocular chitin, and is hollow, its cavity being occupied by a vitreous concretion (Text-fig. 20 D). A firm connexion of this kind might be an effective means of transmitting any motion of the chitin to the underlying epithelium.

The few observations that I have made on the development of the pseudoculi are described in section 10 (v).

(iv) *The Basal Antennal Sense Organs*. Between the bases of the antennae are a pair of very pronounced thickenings of the epidermis, each connected to the deutocerebrum by a thin nerve that lies well above the main antennal nerve (Text-figs. 16, 17). Although located mainly outside the antenna, these epidermal thickenings intrude a little into the basal antennal segment, to the wall of which they are attached (Text-fig. 16 A).

Such an organ might be expected to respond to movement of the antenna as a whole, recalling, in this respect, the peculiar organ of Johnston of the pedicellus of the insect antenna.

They arise as local thickenings of the epidermis in very advanced embryos (fig. 102, Pl. 9). Their nerves probably grow into the brain along the tergal adductor muscle of the antenna, alongside which they lie.

(v) *The Exsertile Vesicles* (?) *of the Collum Segment*. These peculiar structures are generally regarded, though for no good reason, as the vestigial appendages of the first abdominal segment. Schmidt (1895), who first suggested this, had, however, already recognized their possible affinity with the exsertile vesicles (coxal sacs) of Symphyla.

They are borne on a pair of gentle swellings on the floor of the collum segment, beyond which they protrude towards one another, ending in a slight button-like expansion (Text-figs. 2 B, 24). Each organ appears as a compact bulbous swelling of the epidermis that intrudes well into the body-cavity. It consists of five or six large cells, without obvious cell-boundaries. Their cytoplasm is, for the greater part, granular; but toward the free end it acquires a peculiar hyaline texture, and, unlike the rest of the organ, stains very deeply (Text-fig. 21). The exposed tapering end of the organ is invested in chitin, which becomes thinned out and reduced to a just perceptible sheath on the button-like expansion. The nature of these peculiar structures is not known with certainty. It has been suggested that they may be sense-organs; there is, however, no clearly recognizable nerve connected with them. It seems more probable that they should be compared with the exsertile vesicles of certain other myriapods and primitive insects; but it should be noted that there is no muscle connected with them, though a thin muscle is attached to the chitin immediately to their rear (Text-fig. 17).

They develop from vestiges of the 'ventral organs' of the collum segment, and not out of appendage-Anlagen. In embryos aged about 10 days these 'ventral organs' may be seen in progress of absorption into the collum ganglion, which has itself now separated almost completely from the epidermis (fig. 95, Pl. 8). Sections from more advanced embryos, taken at exactly the same level, show that a small group of cells from each 'ventral organ' has

remained within the sternal epidermis, and from here intrude into the body-cavity (fig. 96, Pl. 8). Out of them the exsertile vesicles will develop. In early pupae the cells and their nuclei are seen to enlarge, and acquire a granular texture of cytoplasm (fig. 97, Pl. 8). Thereafter their lower tapering ends begin to protrude beyond the surrounding epidermis, and form the peculiar button-like flattening above referred to. They now differ from the adult organs only in their smaller size.

The gentle swelling on the floor of the collum segment, on which these organs are borne, may readily be seen in advanced embryos (fig. 30 A, Pl. 3).
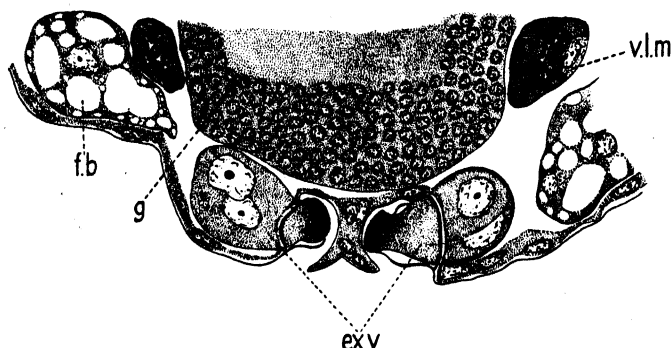
TEXT-FIG. 21. Transverse section through floor of collum segment, from a second instar larva, showing structure of 'exsertile vesicle'.

Lettering. ex.v exsertile vesicle; *f.b* fat-body; *g* collum ganglion; *v.l.m* ventral longitudinal muscle.

In Symphyla, where there is a succession of well-developed exsertile vesicles along the abdominal segments, their derivation from the 'ventral organs' of the embryo is clear (Tiegs, 1940). The similar origin of the organ of the collum segment of *Pauropus* is additional evidence of affinity with exsertile vesicles, and is conclusive evidence against their supposed homology with appendages, for these arise in a position lateral to the 'ventral organs'.

(vi) *The Coxal Apodemes and the Hypopharyngeal Apophyses*. The coxal apodemes, which are shown in Text-fig. 15, are thin, backwardly directed chitinous ingrowths from the anterior margin of each coxa, and are connected at their hinder ends with intersegmental attachments of the ventral longitudinal muscles. They arise in the advanced embryo as simple epidermal ingrowths from the bases of the coxae, and become chitinized shortly before the larva emerges (fig. 107, Pl. 9).

The structure of the hypopharyngeal hypophyses is described in section 6 (ii) (*b*). They begin to form on about the ninth day, as a pair of ingrowths of epidermal cells, just anterior to the mandibles, and a little median to the pre-mandibular glands. In the absence of intersegmental lines it is impossible to determine accurately their segmental allocation; but their position immediately in front of the mandibles makes it probable that they are actually

of intersegmental origin, having grown in along the intersegmental line between the mandibular and pre-mandibular segments. During the tenth day they grow back into the cavity of the head, where they may be seen in a groove between the pre-mandibular and mandibular ganglia, just median to the ingrowing mandibular apodemes (fig. 115 A, Pl. 9). In sections cut 'horizontally' through the head they are found to have bent round the ganglia on to the (posteriorly directed) superior surface of the latter (fig. 94 D, Pl. 8). In contact with them, at their sides, is the mesoderm of the mandibles.

Before the end of the tenth day the hinder tips of the ganglia have become connected, behind the pre-mandibular ganglia, with the mesoderm cells from the oesophagus; out of these mesoderm cells, which may be seen in fig. 116 B, Pl. 10, will develop the oesophageal dilator muscles. Already at this time the ascending arms of the apophyses have begun to form; the latter may be seen in fig. 116 A, Pl. 10, growing up over the lateral surface of the brain on to the dorsal head-wall, and it will be evident from the section (which immediately precedes fig. 116 B in the series) that the ascending arms develop at only a short distance from the hinder tips of the apophyses.

In the advanced embryo a short string of cells has appeared connecting the mandibular apodemes with the hypopharyngeal apophyses (fig. 117, Pl. 10), but from which of these two structures they have arisen I am unable to state. In them we see the Anlage of the median ligament of the mandible (see section 6 (ii) (b)).

In young pupae the apophyses are still two separate cords of cells that bend round the tritocerebrum from their point of origin just in front of the mandibles to their attachment to the oesophageal dilator muscles, and with the ascending arm arising from near their hinder ends (fig. 119, Pl. 10). But as the pupa matures the ganglia recede considerably in the head (cf. Text-figs. 8, 9), enforcing elongation of the apophyses, which now enter the collum segment; and this elongation takes place mainly behind the point of origin of the ascending arms. The oesophageal dilator muscles, hitherto transversely disposed (fig. 117, Pl. 10), are thereby drawn into a position in which they lie more and more parallel with the oesophagus; this is, indeed, already seen in fig. 119, Pl. 10. With the enlargement of the apophyses, their bases approach one another below the oesophagus, where they finally fuse (fig. 120, Pl. 10); in this way the ring round the oesophagus begins to develop.

In these late pupae the apophyses are seen to be continued into two diverging arms of cells that lie in front of the mandibles. From fig. 120, Pl. 10, it is evident that they form part of the wall of the pre-oral cavity, and that they are the suspensorium of the hypopharyngeal apophyses (see section 6 (ii) (b)). They should probably be assigned to the pre-mandibular segment.

Shortly before the larva is due to emerge the apophyses develop an inner core of chitin.

(vii) *The 'Dorsal Organ'*. An unpaired 'dorsal organ' is present in the embryo of *Pauropus*, being located in the provisional body-wall mid-dorsally between the anterior and posterior tips of the germ-band.

There is much individual variation in the time of its appearance; the large

cells out of which it will form are sometimes distinguishable as early as the advanced gastrula, while in exceptional cases even late blastoderms are met with in which they are not yet recognizable.

A section through a late gastrula, with the 'dorsal organ' in course of formation, is shown in fig. 46, Pl. 4; a group of five 'yolk-pyramids' (of which two only are present in the section) are here distinguishable by the great size of their nuclei, which, unlike those of the adjacent blastoderm, still lie embedded in the yolk. On the other hand, in the early blastoderm shown in fig. 47, Pl. 4, such cells are not even present. But in advanced blastoderms they may almost always be seen. Below them the intravitelline protoplasmic reticulum is unusually dense, and extends even as far as the central clump of endoderm (figs. 48, 49, Pl. 4; fig. 53, Pl. 5). Throughout the whole course of development of the 'dorsal organ', its cells remain, without cell-walls, in direct continuity with this intravitelline protoplasm.

Probably in consequence of individual differences in the time of first appearance of the 'dorsal organ', sections through the immature organ do not present any uniform picture. Sometimes the large nuclei lie well below the surface of the egg, embedded in yolk (fig. 48, Pl. 4); and here we are probably concerned with an organ which has begun to develop prematurely in the gastrula. In other cases the enlarged cells are a portion of the blastoderm itself, and give no evidence of having arisen within the yolk (fig. 49, Pl. 4). Eventually, however, there is formed, in all cases, a circular disk of enlarged cells which have their long axes towards the middle of the disk (fig. 55, Pl. 5); their cytoplasm is granular and suggests a secretory function. This circular disk of enlarged cells is sometimes, as in the case here illustrated, present as early as the blastoderm stage; but more frequently it does not appear till well after the germ-band has formed.

In this condition the organ does not long remain, for it soon intrudes more deeply into the yolk, while a depression develops at the surface in which a secretion then begins to appear (fig. 56, Pl. 5; Text-fig. 5). This soon increases in quantity, filling the gradually deepening hollow in the middle of the disk, and slowly spreading from there outwards underneath the blastodermic cuticle (fig. 57, Pl. 5).

During the eighth day the organ shows signs of becoming constricted off from the surrounding epidermis (Text-fig. 7; fig. 103, Pl. 9), and eventually loses connexion with the latter, its cells undergoing disruption in the yolk. The secretion is usually visible for a few days longer, spreading for a little distance from the site of the former organ beneath the blastodermic cuticle, to which it probably serves to attach the embryo.

Students of arthropod development have long been familiar with the occurrence of 'dorsal organs' and paired 'dorso-lateral organs' in the embryos of some of the most diverse members of the phylum. There can be little doubt that the organs referred to by this name are not all homologous, and structurally they may differ widely. The Symphyla, Collembola, and *Campodea* are distinguished by the possession of an unusually remarkable organ, for it is the

source from which a system of extra-embryonic filaments radiate, sometimes for long distances, over the surface of the embryo (Tiegs, 1942 *a*, 1942 *b*). The presence of similar organs in the embryo of *Pauropus* would not have caused surprise; as it has turned out, however, the most singular feature of this type of organ—the system of extra-embryonic filaments—is lacking. Neither in Diplopoda nor Chilopoda is a 'dorsal organ' of this type known, and it may, for the present, be regarded as limited to Symphyla and primitive insects.

The 'dorsal organ' of *Pauropus* recalls, in one respect, the paired 'dorsolateral organs' of certain crustacean embryos. In *Idotea* these organs have a glandular function, their secretion probably serving to attach the embryo to the egg-shell (Nusbaum and Schreiber, 1898). In *Hemimysis* the organ is also secretory (Manton, 1928). In both cases it is purely transitory, and degenerates within the yolk.

15. *The Muscular System*

(a) *Musculature of Adult*

Silvestri (1902) has already given a good account of the principal muscles of *Allopauropus brevisetus*. His description, however, omits the muscles of the mouth-appendages, on which information is specially needed. For the purpose of the present work I have made a more complete examination of the adult musculature, which on the whole has confirmed Silvestri's work.

The tergal muscles of the head (Text-fig. 22 B, D) comprise: (i) Two long muscles (*t.ab.a*, *t.ad.a*), which pass forward from the occipital suture, and are attached to the base of the antenna, on which they exert an abductor and an adductor function respectively; (ii) a very large dorsal-longitudinal muscle (*d.l.m.h*), lying lateral to these muscles, originating from the occipital suture, and attached in front to the wall of the head-capsule, lateral to the antenna; this peculiar muscle has, for its probable function, the adjustment of convexity of the chitin of the pseudoculus; (iii) two large muscles originating from the roof of the head-capsule and inserted on to the mandibular apodeme, one (*t.rt.mn*) running forward, and acting probably as a retractor on the mandible, while the hinder and shorter muscle (*t.d.mn*) is probably a depressor; (iv) a long and narrow levator (?) of the maxilla (*t.l.mx*), taking origin from the occipital suture, and inserted on to the stipes of the maxilla; (v) a narrow retractor (?) of the maxilla, arising from the side of the head, and attached to the maxillary apodeme (*t.rt.mx*; Text-fig. 22 A).

Most of the sternal muscles of the head-appendages take origin from the hypopharyngeal apophyses. They comprise (Text-fig. 22 A, C): (i) Two narrow muscles (*s.r.a*) to the basal segment of the antenna, upon which, according to Silvestri, they may exert a rotator effect; (ii) a strongly developed set of muscles to the mandible, most of which must act as adductors (*s.ad.mn*); (iii) a muscle which takes origin from the suspensory plate (Text-fig. 2 F) lies parallel with the mandible and probably acts as protractor (*s.pr.mn*); (iv) a long and narrow muscle (*s.pr.mx*) arising from the apophysis and attached to the hinder end of the maxillary apodeme, and functioning probably as a protractor. Associated

with the maxilla are at least two small muscles: (i) a flexor muscle passing from the stipes to the lacinia (*f.l*); (ii) a very thin sternal depressor (?) arising from the floor of the head (*s.d.mx*; Text-fig. 22 B).

The muscles associated with the fore-gut and pre-oral cavity are (Text-figs. 17, 23 A, C): (i) Two sets (outer and inner) of short buccal dilator muscles (*b.d.m*), the m. elevatores palati of Silvestri, which pass from the roof of the pre-oral cavity to the clypeus; (ii) a pair of muscles that arise from the lateral margins of the intermaxillary plate, and converge on to the floor of the pre-oral cavity, on which they act as depressors (*b.dp*); (iii) a short retractor to the clypeus (*r.c*), arising from the hypopharyngeal apophysis; (iv) a set of very strongly developed oesophageal dilator muscles (*oes.d.m*), which take origin from the hindermost end of the apophysis, and pass forward to become attached to the floor of the oesophagus; the remarkable development of these muscles suggests a strong suctorial function for the oesophagus (see also Text-fig. 16 B).

It should be noted that there are no muscles arising from the dorsal arm of the hypopharyngeal apophysis; this arm is attached by fibrous tissue to the occipital suture, and evidently imparts stability to the apophysis.

The musculature of the antenna (Text-fig. 22 G) comprises a number of short flexor (*f.a*) and extensor (*ex.a*) muscles, passing between the segments, as well as a flexor (*f.r*) and extensor (*ex.r*) muscle for each of the two rami.

The trunk musculature, as might be expected for such active animals, is strongly developed. The following sets of muscles may be identified (Text-fig. 22 C, D, E): (i) the dorsal longitudinal muscle (*d.l.m*), comprising some relatively long muscles that run from the middle of one tergal shield to the middle of the next, as well as some shorter slightly diverging muscles that pass forward or backward from the middle of one tergite to the posterior or anterior margin respectively of the adjacent tergites (see Text-fig. 22 D); (ii) a set of oblique abdominal muscles (*obl.a*) which pass downwards from the anterior margins of the tergal shields, and become attached at the intersegments, one or two segments to the rear; (iii) a set of oblique abdominal muscles (*obl.b*) which pass upwards from their intersegmental attachments, and become connected to the hinder margins of the tergal shields, usually of the succeeding segment (the most anterior of these acts as a retractor of the head—*rt.h*); (iv) simple dorso-ventral muscles (*d.v.m*) that pass down from the tergal shields to become connected at the intersegments, the fourth, sixth, eighth, and tenth segments, which are devoid of tergal shields, utilizing the preceding tergal shields for the dorsal attachment of these muscles; (v) the ventral longitudinal muscles (*v.l.m*), segmentally disposed, and attached at successive intersegments, the most anterior of this set being attached to the hypopharyngeal apophyses; (vi) a set of crossed sternal muscles (*c.s.m*), which pass back from one intersegment to the succeeding intersegment of the other side (Text-fig. 22 E).

Apart from these muscles there are also three large muscles that exert an action on the head (Text-fig. 22 C). These are: (i) a rotator (*r.h*) arising from
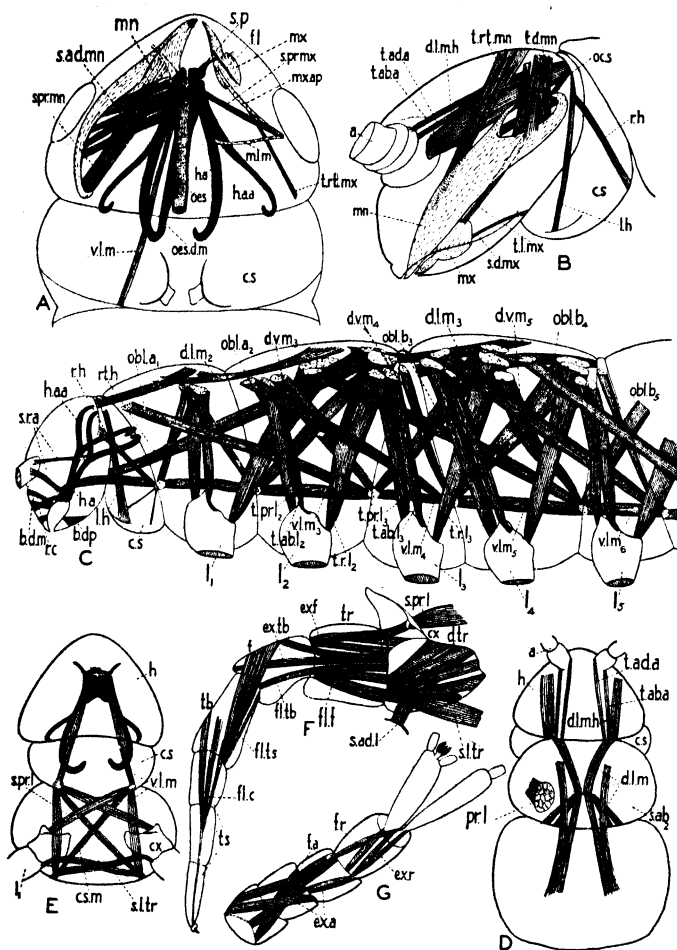
TEXT-FIG. 22. Muscular System.

A. Head and collum segment, viewed from below; on the left side has been drawn the mandible, on the right the maxilla only. B. Head and collum segment in lateral view. C. Trunk musculature, lateral view; the numerals attached to individual trunk-muscles are for comparison with Text-fig. 25. D. Tergal muscles, viewed from above. E. Sternal muscles, viewed from below. F. Right leg, in ventral view. G. Antenna. The key to the lettering of the muscles is given in the text.

Additional lettering: *a* antenna; *c.s* collum segment; *cx* coxa; *f* femur; *h* head; *h.a* hypopharyngeal apophysis; *h.a.a* ascending arm of latter; l_1–l_5 first to fifth legs; *m.l.m* median 'ligament' of mandible; *mn* mandible; *mx* maxilla; *oc.s* occipital suture; *oes* oesophagus; *s.ab₂* second abdominal segment; *s.p* suspensorial plate; *tb* tibia; *ts* tarsus; *tr* trochanter.

the hinder margin of the collum segment; (ii) a levator (*l.h*) arising from the floor of the collum segment (see also *l.m.h*, Text-fig. 17); (iii) a long and powerful retractor (*rt.h*), arising from the hinder margin of the second tergal scute, and attached to the hinder margin of the head (this muscle is, as Text-fig. 22 C shows, the first member of the second oblique series); (iv) two pairs of thin muscles passing to the floor of the collum segment (shown in Text-fig. 22 C, but not labelled).

The extrinsic musculature of the leg comprises the following muscles (Text-fig. 22 C, E): (i) three tergal muscles for each leg, namely, a large pro-motor (*t.pr.l*), a weaker abductor (*t.ab.l*), and an exceptionally large backwardly directed re-motor (*t.r.l*); (ii) two sternal muscles arising intersegmentally from the opposite side, one muscle being attached to the trochanter of the leg and acting evidently as a levator (*s.l.tr*), while the other muscle is attached to the apodeme of the coxa, and is probably a pro-motor of the leg (*s.pr.l*); (iii) a short adductor (?) from the apodeme to the coxa (*s.ad.l*) (Text-fig. 22 F).

The intrinsic muscles of the leg have already been described by Silvestri (1902) and Ewing (1928). Both these authors have shown that there are no muscles arising within the last three segments of the leg, which, following Ewing, we may therefore regard as tarsus. The muscles of the leg are shown in Text-fig. 22 F. The principal muscles of the coxa comprise a strongly developed system of depressor muscles for the trochanter (*d.tr*); I cannot recognize a levator for the trochanter, other than the large sternal levator which arises from the opposite intersegmental apodeme. The remaining muscles of the leg comprise: (i) a strongly developed system of flexors and extensors for the femur (*fl.f; ex.f*); (ii) extensor and flexor muscles for the tibia (*ex.tb; fl.tb*); (iii) a large flexor for the tarsus (*fl.ts*), an extensor being apparently absent; (iv) a muscle inserted by a long tendon on to the claw, and acting as a flexor of the claw and of the tarsus (*fl.c*).

(b) Development

(i) *Muscles of the head.* The tergal muscles of the antenna and the dorso-lateral longitudinal muscle of the head arise from the antennary mesoderm. After breakdown of the antennary somite, the hollow of the antenna becomes filled with an unorganized mass of mesoderm cells which, during the ninth day, spreads as an irregular column of cells from the base of the appendage backward along the dorso-lateral head-wall to the side of the brain (fig. 110, Pl. 9). From this mesoderm a mass of cells separates off below the base of the antenna, and becomes the rudiment of the dorso-lateral longitudinal muscle; other cells spreading back along the roof of the head may be seen, in very advanced embryos, in process of conversion into the tergal muscles of the antenna. In newly formed pupae muscle fibres are already distinguishable.

The sternal muscles of the antenna and of the mouth-appendages develop in intimate association with the hypopharyngeal apophyses. The latter, as already described in section 14 (vi), begin to develop during the ninth day as a pair of ectodermal ingrowths, immediately in front of the anterior margin of

the mandibles. They grow backwards into the cavity of the head, where, during the tenth day, they may be seen lying to the side of the mandibular ganglion. Their relation to the latter, and to the mesoderm of the antennary and gnathal segments, will be understood by reference to figs. 94 D and 115 A, B, Pl. 9, fig. 115 being drawn from a 10-day embryo, and representing a frontal section through the head, while fig. 94 D is a 'horizontal' section through a similar embryo, just above the bases of the mandibles. It will be seen that each apophysis lies just median to the main mass of the mandibular mesoderm, which is being drawn into the cavity of the head with the ingrowing mandibular apodeme. Mesoderm cells from the hinder margin of the base of the antenna lie in close proximity, a little dorso-laterally to the mandibular apodeme, while from below mesoderm from the maxillary segment is growing upward. The relation of the latter to the apophysis is not clear from the section shown in fig. 115 A, Pl. 9, but if we examine the section immediately to the rear (fig. 115 B), it is at once evident that maxillary mesoderm is being drawn in with the ingrowing maxillary apodeme, just behind the mandibular apodeme, into the direction of the apophysis.

The apophysis is thus in close proximity to the mesoderm of all three head-appendages. In sections through later embryos we find that a direct connexion between this mesoderm and the apophysis has been established (fig. 112, Pl. 9). Out of this will form the sternal musculature of the appendages, their differentiation into muscle-fibres taking place in the advanced embryo and pupa. The development of the sternal muscles of the antennae is necessarily attended by a considerable change in their direction, as these appendages gradually move on to the anterior surface of the head.

The tergal muscles of the mandibles arise from cells that pass from the hinder tips of the mandibular apodemes on to the head-wall, i.e. purely out of mandibular mesoderm. I have not been able to observe the origin of the tergal muscles of the maxilla. (It must be evident, from the manner of their formation, that the attachment of the tergal mandibular and antennary muscles on the wall of the head cannot provide a guide to defining their segments in the definitive head-capsule.)

The development of the large oesophageal dilator muscles has already been described in section 14 (vi).

(ii) *Muscles of the abdomen.* Except for the system of dorsal longitudinal muscles, the abdominal musculature takes its origin wholly from cells that are released by disruption of the abdominal somites.

The Anlage of the dorsal longitudinal musculature is distinguishable very early, for it never forms part of the mesodermal somites. It has already been referred to in section 7. In 7-day embryos, after the somites have begun to form, a pair of irregular bands of mesodermal cells may be seen along the lateral walls of the embryo, dorso-lateral in position to the row of somites (fig. 64, Pl. 5). As far as I have been able to observe, all the segments from the maxillary to the fifth abdominal contribute to their development. In sections cut 'horizontally' along the embryo, the cells in these bands appear irregularly

clumped, but they do not show any obvious sign of segmentation, such as the somites do (fig. 72, Pl. 6). By the ninth day these cells have usually aggregated into four distinct masses (fig. 73, Pl. 6), though sometimes even in older embryos an irregular alignment of cells still persists (fig. 94 B, Pl. 8). The most anterior and least well-defined of these masses lies a little behind the antennary somite, and the hindermost in the fifth abdominal segment. They are evidently the Anlagen of the four dorsal longitudinal muscles of the larva. This clumping of the dorsal longitudinal mesoderm into four masses of cells may not be looked upon as an expression of primary segmentation, equivalent to that of the somites, for all the dorsal longitudinal muscles are not strictly segmental in location (cf. Text-fig. 22 C). In later embryos the muscle-Anlage moves into a still more dorsal position (figs. 106, 107, 109, Pl. 9), and there undergo differentiation into muscle tissue.

The disruption of the abdominal somites during the ninth day is attended by a re-grouping of cells, in which the Anlagen of certain of the abdominal muscles become, for the first time, apparent. Of these the most conspicuous are the ventral longitudinal muscles. They soon become recognizable in each segment as a pair of narrow bands of cells, lying alongside the nerve-cord (fig. 78, Pl. 7). They are present in all the abdominal segments except the anal. In the adult animal the most anterior of these muscles is attached to the hypopharyngeal apophysis (Text-fig. 22 C), and it would therefore seem that the gnathal segments of the head contribute some myoblasts to the ventral longitudinal musculature. In sections through later embryos these muscle-Anlagen are exceedingly prominent to the side of the nerve-cord, where they are now undergoing conversion into muscle tissue (figs. 106, 107, Pl. 9). They have by this time acquired intersegmental attachments. In pupae they are seen in a more ventral position relative to the nerve-cord (fig. 108, Pl. 9; also fig. 109, Pl. 9, from a larva).

Spreading down from the base of each appendage, under the ventral longitudinal muscle, is another group of mesoderm cells, out of which the sternal musculature will develop (fig. 78, Pl. 7). In the advanced embryo these cells grow under the nerve-ganglia, after these have separated from the ventral epidermis; their conversion into muscle-fibres begins in the late embryo, even before the pupa forms.

The various dorso-ventral and oblique muscles of the abdomen all arise from cells which spread up from the bases of the legs over the lateral epidermis. They may be seen in fig. 106, Pl. 9. Their conversion into muscle-fibres takes place in the advanced embryo (fig. 107, Pl. 9).

The musculature of the legs develops out of the clumps of mesoderm that occupy the hollows of the limb-buds, becoming drawn out with the latter when, in the late embryo, these elongate to form the legs (fig. 106, Pl. 9).

DESCRIPTION OF PLATES

PLATE 1

Fig. 1. Fragment of adult ovary, cut in 'horizontal' section. The germarium lies between the two rows of oocytes that are enlarging along the sides of the ovary. Three of its cells (indicated by x) are in prophase of meiosis; one (y) is in mitosis; others (z) are in resting phase. But within the germarium are also young oocytes, in an initial phase of enlargement, and with nuclei in the germinal vesicle condition. $\times 770$.

Fig. 2. Fragment of a sagittal section along ovary, in which some of the oocytes have begun to accumulate yolk. There are also three enlarging oocytes still without yolk, and in one of these a 'yolk-nucleus' is seen. $\times 390$.

Fig. 3. Polar view of the chromosomes, from an ovarian egg shortly before laying; first meiotic metaphase. The full set of 13 bivalent chromosomes ('tetrads') is shown. $\times 1,600$.

Fig. 4. Similar stage, showing spindle fibres. Only 6 of the 13 bivalents are present in the section. $\times 1,600$.

Fig. 5. From an almost newly laid egg. The chromosomes and their enveloping cytoplasm have moved to the periphery. Nine of the 13 bivalents, still showing 'tetrad' formation, and still located in a single plane, are present in the section. $\times 1,600$.

Fig. 6. Anaphase of first meiotic division. $\times 1,600$.

Fig. 7. The same; polar view. $\times 1,600$.

Fig. 8. Fragment of a tangential section at the surface of the egg, showing first and second polar bodies; from an egg in which fusion of male and female pro-nuclei has already taken place. $\times 1,600$.

Fig. 9. Portion of a section through an egg, showing male and female pro-nuclei just prior to fusion. $\times 950$.

Fig. 10. Section through egg after fusion of pro-nuclei. A true resting nucleus has formed. $\times 710$.

Fig. 11. Egg with 2 blastomeres. $\times 480$.

Fig. 12. Egg with 4 blastomeres. In this egg the cleavage grooves between the blastomeres are unusually deep. $\times 480$.

Fig. 13. Egg with 6 blastomeres. $\times 480$.

Fig. 14. Egg with 8 blastomeres. $\times 480$.

Fig. 15. Egg with 16 nuclei, but in which only 11 blastomeres have so far become demarcated. $\times 480$.

Fig. 16. Egg with 24 blastomeres (early blastula stage). $\times 480$.

Fig. 17. Egg with about 40–5 cells (late blastula). $\times 590$.

Fig. 18. Egg with about 80 blastomeres (early gastrula). $\times 590$.

Fig. 19. Egg with about 200 cells (late gastrula). $\times 700$.

PLATE 2

All drawings in this plate are from stained whole embryos.

Fig. 20. Mature blastoderm. $\times 380$.

Fig. 21. Early phase in the differentiation of the germ-band out of the blastoderm; stage with ventral aggregation of nuclei and cytoplasm. $\times 380$.

Fig. 22. Early germ-band; lateral view. $\times 380$.

Fig. 23. Oblique view of an embryo rather more advanced than that shown in the previous figure. The head-lobes have begun to form, and the stomodaeal opening has appeared. $\times 380$.

Fig. 24 A, B. Lateral (left) view and anterior view respectively of a 6-day embryo. The antennae are developing; the stomodaeum is now conspicuous. $\times 380$.

Fig. 25 A, B. Similar views of a 7-day embryo. The rudiments of another pair of appendages (mandibles) have formed. On the head-lobes the invaginated posterior lobes of the protocerebral ganglia have appeared. $\times 380$.

Fig. 26 A, B. Lateral (right) and anterior views of a more advanced 7-day embryo, in which the appendage rudiments as far back as the first legs have appeared. $\times 380$.

Fig. 27 A, B. Similar views of an embryo aged about 8 days, showing two well-developed leg-rudiments, and the third in course of formation. The embryo shows the initial stage in development of the pre-oral cavity. $\times 380$.

PLATE 3

Fig. 28 A, B. Lateral (left) and anterior views, respectively, of a 9-day embryo. × about 420.

Fig. 29 A, B. Lateral (right) and antero-ventral views respectively of an embryo aged fully 10 days. Fig. A × 430; Fig. B × 380.

Fig. 30 A, B. A. Antero-ventral view of an embryo, shortly before hatching.

B. View into the pre-oral cavity of the same embryo, to show the inturned mandibular sternite; the optical section through the clypeus is represented as a cut edge by lines. A and B × 380.

Fig. 31. Postero-dorsal view of the hinder end of an advanced embryo, at about the stage shown in Fig. 29. In this embryo the outlines of the anal segment have become defined. × 380.

Fig. 32 A, B, C. A. Early pupa (pupal sheath removed). × 370.

B. Right antenna of same, dorsal view. × 800.

C. Second leg. × 610.

Fig. 33 A, B. Advanced pupa. In A is shown the anterior end, seen from the right side, × 600. In B is shown the right antenna in ventral view. × 800.

Fig. 34. Hinder end of a newly emerged first instar larva; lateral view. This drawing, and Figs. 35, 36, 37, are from stained larvae. The investing chitin is shown in outline only. Of the setae, only the large setae of the fifth segment (second trichobothria) are indicated. × 470.

Fig. 35. Hinder end of a more advanced first instar larva, in which the fourth and fifth legs are visible beneath the chitin. The new (seventh) segment is in process of forming; note the Anlage of its sensory seta (trichobothrium). × 500.

Fig. 36. Hinder end of a larva of about similar age, seen from above. × 470.

Fig. 37. Hinder end of an advanced first instar larva shortly before the moult. The fourth and fifth legs are now fully developed; in the new (seventh) segment the new (third) trichobothrium has grown out beneath the chitin, but is not yet fully chitinized. The chitin of the second trichobothrium is being discarded, and a new second trichobothrium is replacing it. × 470.

PLATE 4

Fig. 38. Section through an egg in which the first two cleavage-nuclei have appeared, but in which the partition separating the blastomeres has not yet formed. Note the spindle-fibres between the daughter-nuclei.

Fig. 39. 'Horizontal' section through the 4-cell egg shown in Fig. 12.

Fig. 40. Section through 8-cell egg shown in Fig. 14. Note degenerating polar bodies.

Fig. 41. Section through an egg containing 11 nuclei, of which 7 are in mitosis. Only 4 nuclei are present in this section. Note degenerating polar body within one of the blastomeres.

Fig. 42. Section through 24-cell egg shown in Fig. 16.

Fig. 43. Section through the egg shown in Fig. 17, with 40–5 cells (late blastula stage).

Fig. 44. Section through developing gastrula; about 75-cell stage.

Fig. 45. Section through young gastrula; about 80-cell stage (cf. Fig. 18).

Fig. 46. Section through an advanced gastrula at about the stage of development shown in Fig. 19. This gastrula shows precocious 'dorsal organ' formation.

Fig. 47. Longitudinal section of a young blastoderm.

Figs. 48, 49. Sagittal sections of two mature blastoderms, showing different conditions of development of the 'dorsal organ'. The blastoderms also show the initial stages of migration of yolk-cells into the yolk.

PLATE 5

Fig. 50. Small fragment of a section grazing the surface of a mature gastrula. The drawing represents a transverse section through the outer wall of a single 'yolk-pyramid'. × 800.

Fig. 51. Similar section, from a mature blastoderm. × 800.

Fig. 52. Section through the egg shown in Fig. 21. The section is taken 'horizontally' through the region of thickening, a little below the equator of the egg. Separation of the meso-dermal cells from the anterior (to right) and posterior thickened walls is in progress; the intervening thick epithelium at the sides of the egg does not contribute to the formation of mesoderm. Although endoderm is present in the middle of the egg, neither of its two nuclei lies within the section. × 550.

Fig. 53. Sagittal section of an egg at about the stage shown in Fig. 21. Mesoderm formation is here more advanced than in the egg shown in Fig. 52, for the mesoderm already constitutes a fairly well-defined layer internal to the ectoderm in the lower half of the egg. ×550.

Fig. 54. Sagittal section through an egg with newly formed germ-band (cf. Fig. 22); anterior end to right. The mesoderm is now distinct. The stomodaeum has begun to form. Note the peculiar cytoplasmic inclusions in endoderm. ×550.

Fig. 55. Section through a developing 'dorsal organ' from a late blastoderm. ×800.

Fig. 56. Portion of a transverse section through a 6-day embryo, showing 'dorsal organ'. A depression is forming in its middle, and secretion has begun. ×800.

Fig. 57. Similar section. The depression in the organ is now filled with secretion. ×800.

Fig. 58 A, B. Two sections of a 6-day embryo, from a 'horizontally' cut series. Section B passes through the equator of the egg, section A nearer the upper pole. In A the section passes (below) through the head-lobe, in which the protocerebral ganglionic thickenings are developing; and (above) through the developing proctodaeum. In B it passes (below) a little behind the stomodaeum and (above) through the region of the third abdominal segment. ×800.

Fig. 59. Portion of a section from a 'horizontally' cut series, from a rather later embryo than the foregoing. The section passes through the stomodaeum, and includes the antennary somite, which now lies in line with the stomodaeum. ×800.

Fig. 60. Part of a section through the head of an 8-day embryo at level of antenna (cf. Fig. 67 C, D). The antennary 'ventral organ' has begun to form. In the antennary somite a coelomic cavity is now present. ×800.

Fig. 61. Part of a transverse section through a 7-day embryo, a little behind the stomodaeum, to show the pre-mandibular mesoderm. ×800.

Fig. 62. Fragment of a section from an early 8-day embryo, showing pre-mandibular somite beginning to elongate; the large mass of cells to the right is the inferior wall of the pre-oral cavity. ×800.

Fig. 63. Transverse section through maxillary segment of a 7-day embryo, showing initial stage in formation of the somites. Between the developing somites the 'median mesoderm' is visible. ×800.

Fig. 64. Transverse section of right half of a late 7-day embryo, taken through the maxillary segment. Note, in comparison with Fig. 63, that the maxillary somite has taken form, and is now widely separated from the 'median mesoderm'. Note also the Anlage of the dorsal longitudinal muscle. In the ectoderm we see the first indication of ganglion formation. ×800.

Fig. 65. Transverse section through the collum segment of a 7-day embryo, showing initial stage in formation of somites. Between the two developing somites is a little 'median mesoderm'. Drawn from the same embryo as Fig. 63. ×800.

Fig. 66. Similar section from an 8-day embryo, showing fully formed somite of the collum segment. Note the Anlage of the dorsal longitudinal muscle, quite distinct from the somite. Associated with the collum ganglion is a 'ventral organ'. ×800.

PLATE 6

Fig. 67 A–F. Drawings of six successive sections cut 'horizontally' through an 8-day embryo, to show the somites and developing ganglia of the head. The pre-oral cavity is developing, and it is not possible to delimit it sharply from the stomodaeal ingrowth. In Fig. B the complete section has been drawn, and shows (above) the abdomen transected through its fourth segment; the other five sections show only the head of the embryo. ×870.

In Fig. A the section passes just in front of the stomodaeum and developing pre-oral cavity, and shows the pre-antennary somites which have arisen from the pre-oral mesoderm; to the sides are the pre-antennary ganglia, and to the sides of these the lateral and frontal lobes of the protocerebrum, in which 'ventral organ' cell-disposition is apparent (a fragment of the posterior lobe of the protocerebrum also intrudes into the section).

In B the section grazes the hinder wall of the pre-antennary somites and the anterior wall of the stomodaeum; note the antennary ganglion lying immediately behind the pre-antennary ganglion of the previous section; note also the antenna, and the intrusion of part of its somite into the section.

In c more of the stomodaeum and developing pre-oral cavity appears, and to its right a fragment of the pre-mandibular somite intrudes into the section; the antennary ganglia are present, the left showing 'ventral organ' cell disposition; more of the antennary somite is present.

In D the section passes along the whole length of the stomodaeum and pre-oral cavity, the stomodaeum having extended to the central clump of endoderm; to the side of the pre-oral cavity are the elongating pre-mandibular somites, and, lateral to these, we now see the main bulk of the antennary somite.

In E the elongating pre-mandibular somites are seen, bending round behind the pre-oral cavity; on both sides the mandibular somites have begun to intrude into the section, and on the right side is seen also the tip of the elongating maxillary somite.

In F the main mass of each mandibular somite is now present in the section, and part also of the maxillary somite, which is in process of elongating into the maxillary gland. The hinder-most tip of the pre-mandibular somite intrudes into the section. The large mass of developing ganglion with its 'ventral organ' is the pre-mandibular ganglion. The small clump of ganglion tissue above it is probably mandibular ganglion, intruding from behind into the section (for orientation of the section see Fig. 80).

Fig. 68. Section through the head of an embryo aged about 8 days, taken just in front of the stomodaeum (from a 'horizontally' cut series). The pre-oral mesoderm forms a thick clump of cells, but has not yet developed into pre-antennary somites. To the sides are the strong ectodermal thickenings, from which the pre-antennary ganglia and the lateral and frontal lobes of the protocerebrum will arise, but no 'ventral organ' cell disposition has yet appeared. The drawing should be compared with that shown in Fig. 67 A, which represents a comparable section from a rather more advanced embryo. × 870.

Fig. 69. Fragment of a transverse section through the head of a late 8-day embryo, to show beginning of formation of the left mandibular apodeme, and initial phase in transformation of the mandibular somite. To the right a fragment of the developing pre-mandibular gland intrudes into the section, while to the left is seen a part of the maxillary gland. × 870.

Fig. 70. Similar section to the foregoing, taken from a more advanced embryo, showing right mandible and its developing apodeme; the somite has now disrupted into a clump of myoblasts, from which the mandibular musculature will form. In this section the mandibular ganglion is present; note the remains of its 'ventral organ' forming the floor of the pre-oral cavity. × 870.

Fig. 71. Similar section to that shown in Fig. 62, but from a later embryo in which the pre-mandibular somite is in process of conversion into the pre-mandibular gland. The thickened ectoderm to the left is the mandibular ganglion with 'ventral organ'. × 870.

Fig. 72. Portion of a section cut 'horizontally' through the right half of a late 7-day embryo, showing the Anlage of the dorsal longitudinal muscle. At the upper and lower ends of the section a portion of the fourth abdominal and antennary somites respectively may be seen. × 870.

Fig. 73. Similar section from left half of a 9-day embryo. The Anlagen of the four dorsal longitudinal muscles are now seen. × 870.

PLATE 7

Fig. 74. Transverse section through second abdominal segment of a 7-day embryo, to show initial stage in formation of somites. The section is from the same embryo as are Figs. 63 and 65. × 950.

Fig. 75. Similar section, from a rather older embryo, showing later stage in the formation of the somite. × 950.

Fig. 76. Section through fourth abdominal segment of an embryo a little more advanced than that from which a corresponding section in Fig. 67 B has been drawn. The ganglion-rudiment has enlarged and 'ventral organ' formation has begun. The somites occupy the cavities of the limb bases. In one somite a coelomic cavity is present; the other does not even display a visceral wall. × 950.

Fig. 77. Portion of a longitudinal section along a 9-day embryo, showing beginning of transformation of the somites of the second and third abdominal segments. The somites, still showing a trace of coelomic cavity, are enlarging at their lower ends, and extending into the appendages, of which the bases are present in the section. × 950.

Fig. 78. Section through second abdominal segment of a 9-day embryo. The legs are not present in the section. The somites have disrupted each into a large clump of myoblasts, within which the future ventral longitudinal muscle can already be distinguished. The ganglion is now much enlarged, and four of the ganglion-cells are in mitosis. The 'ventral organs' are at
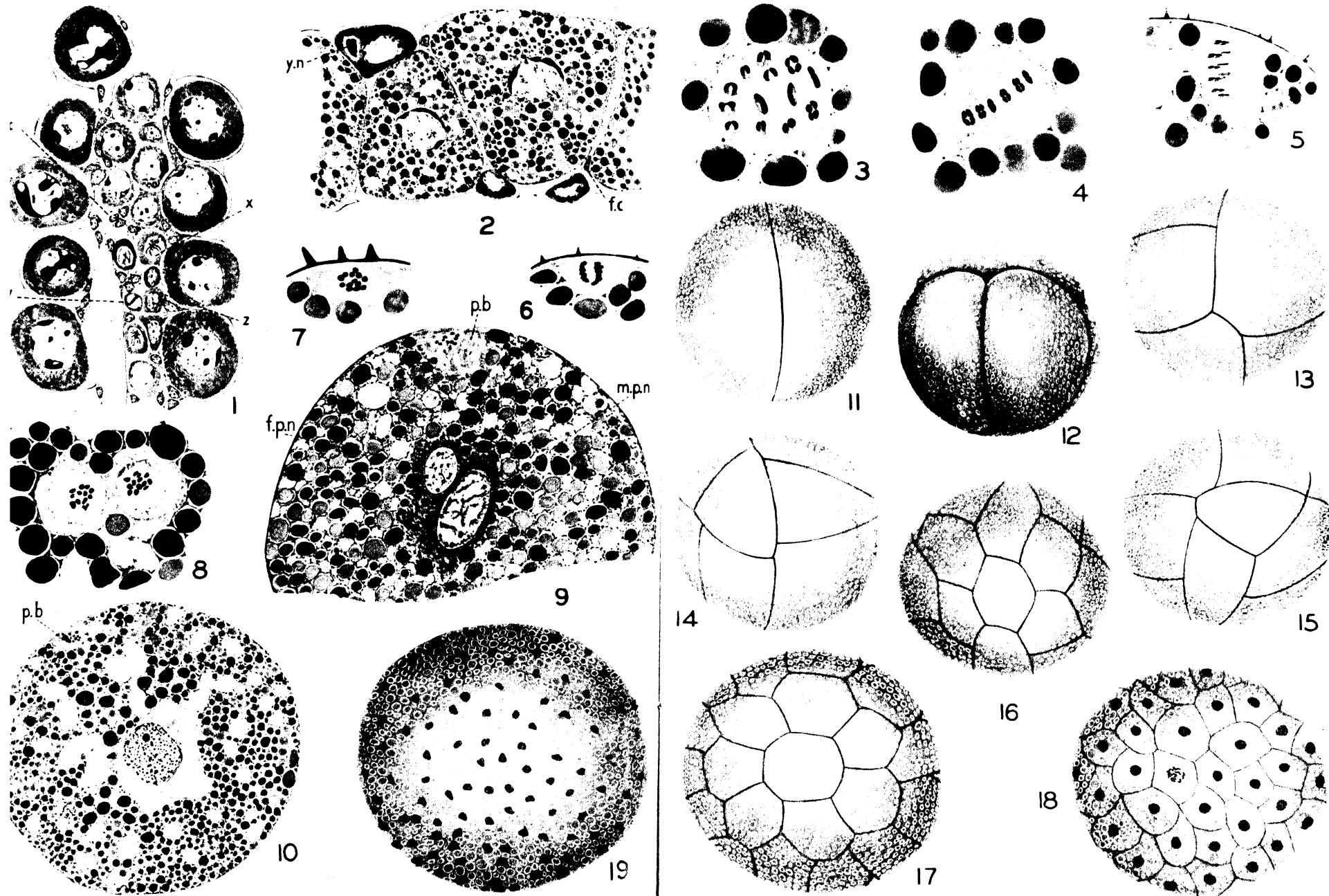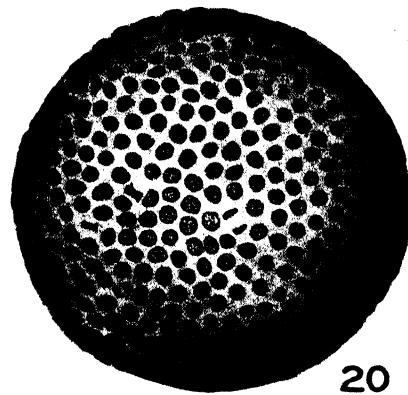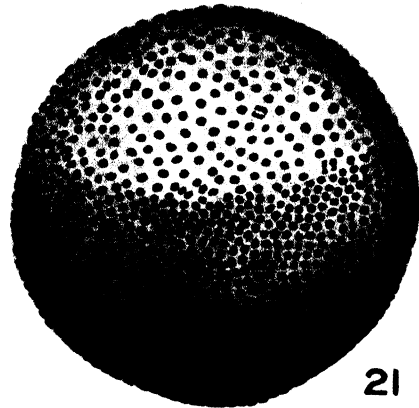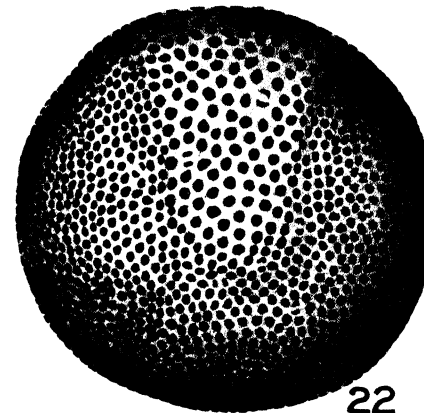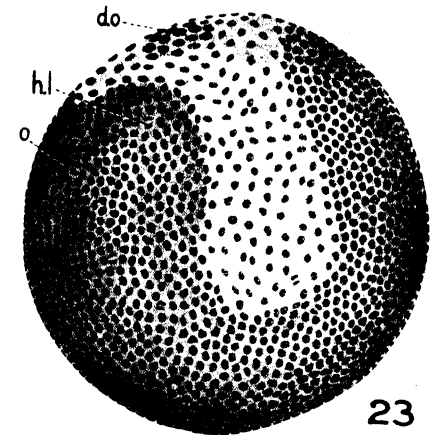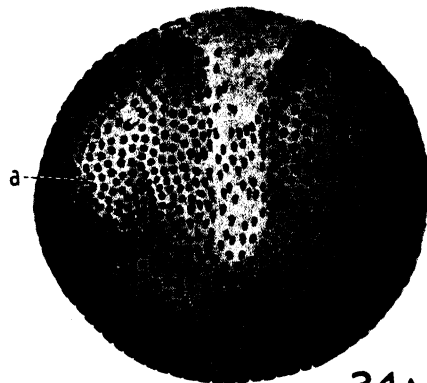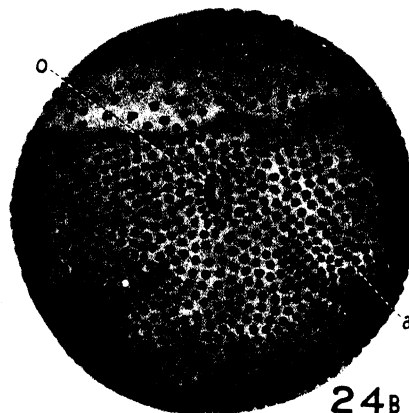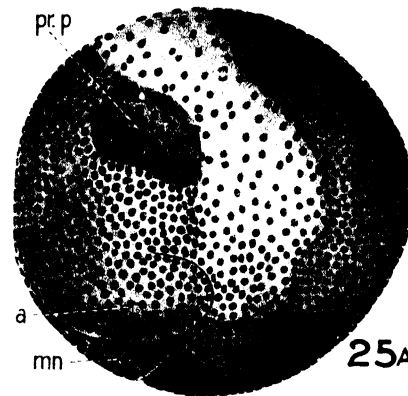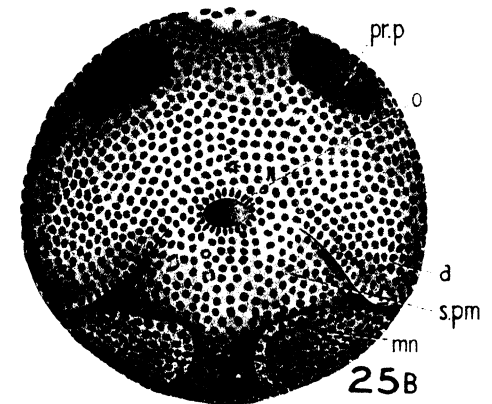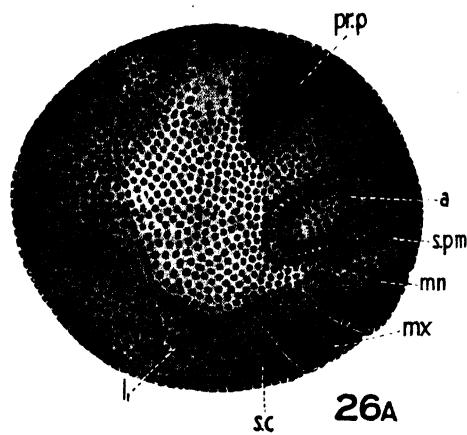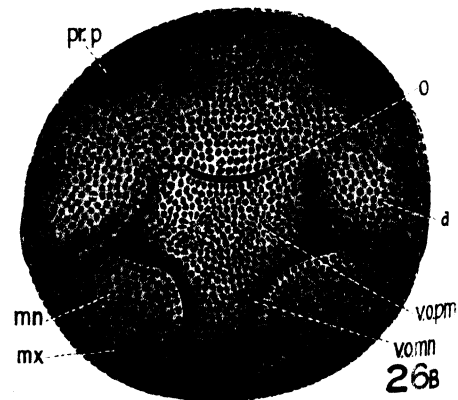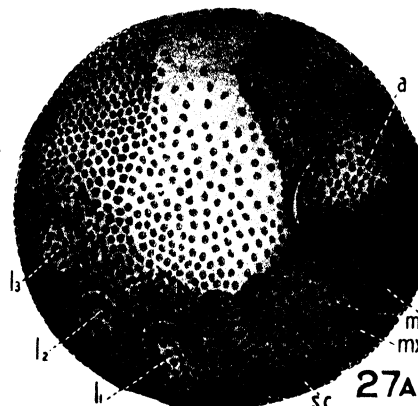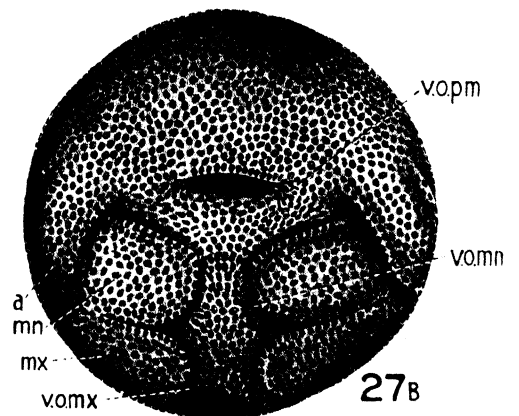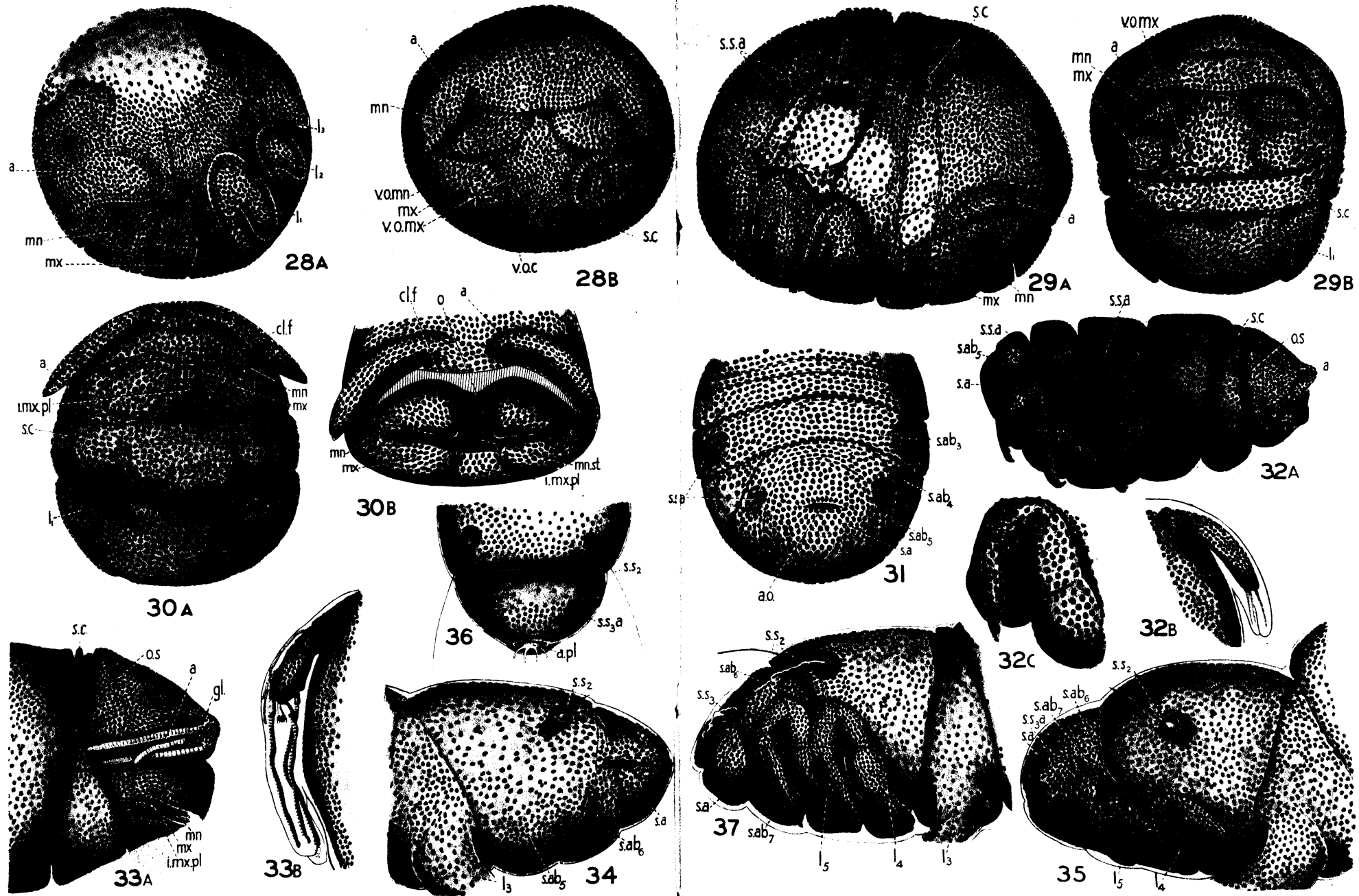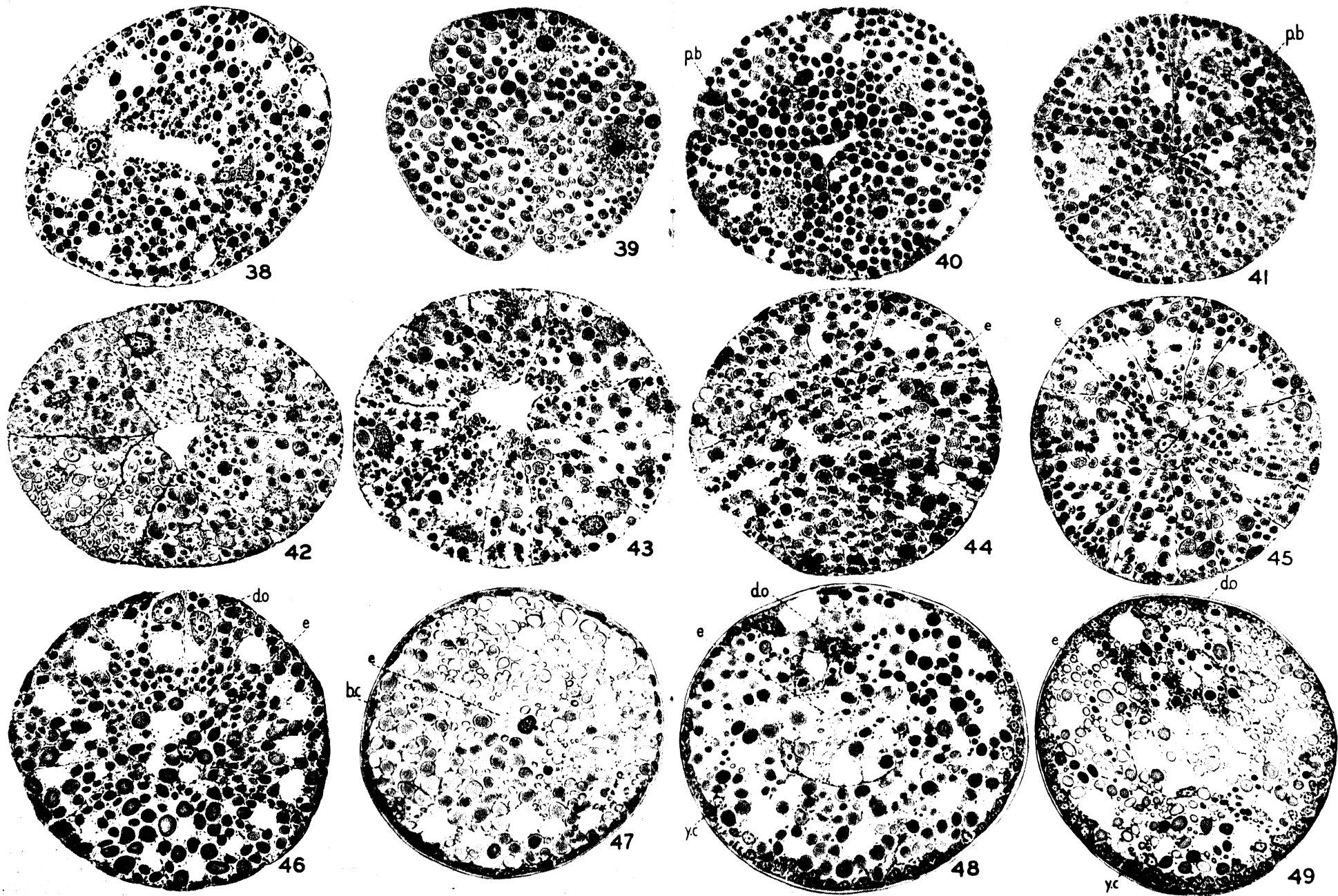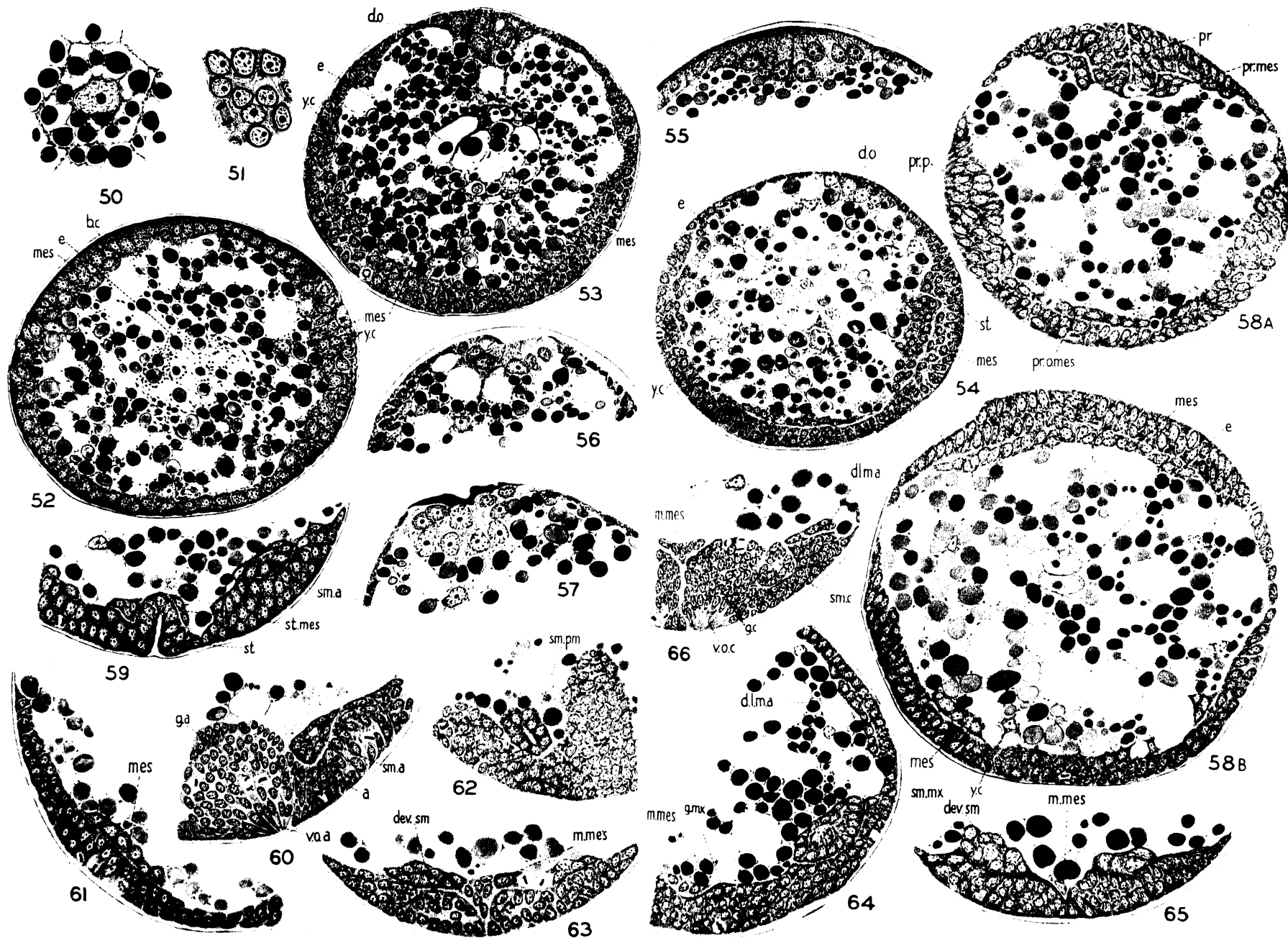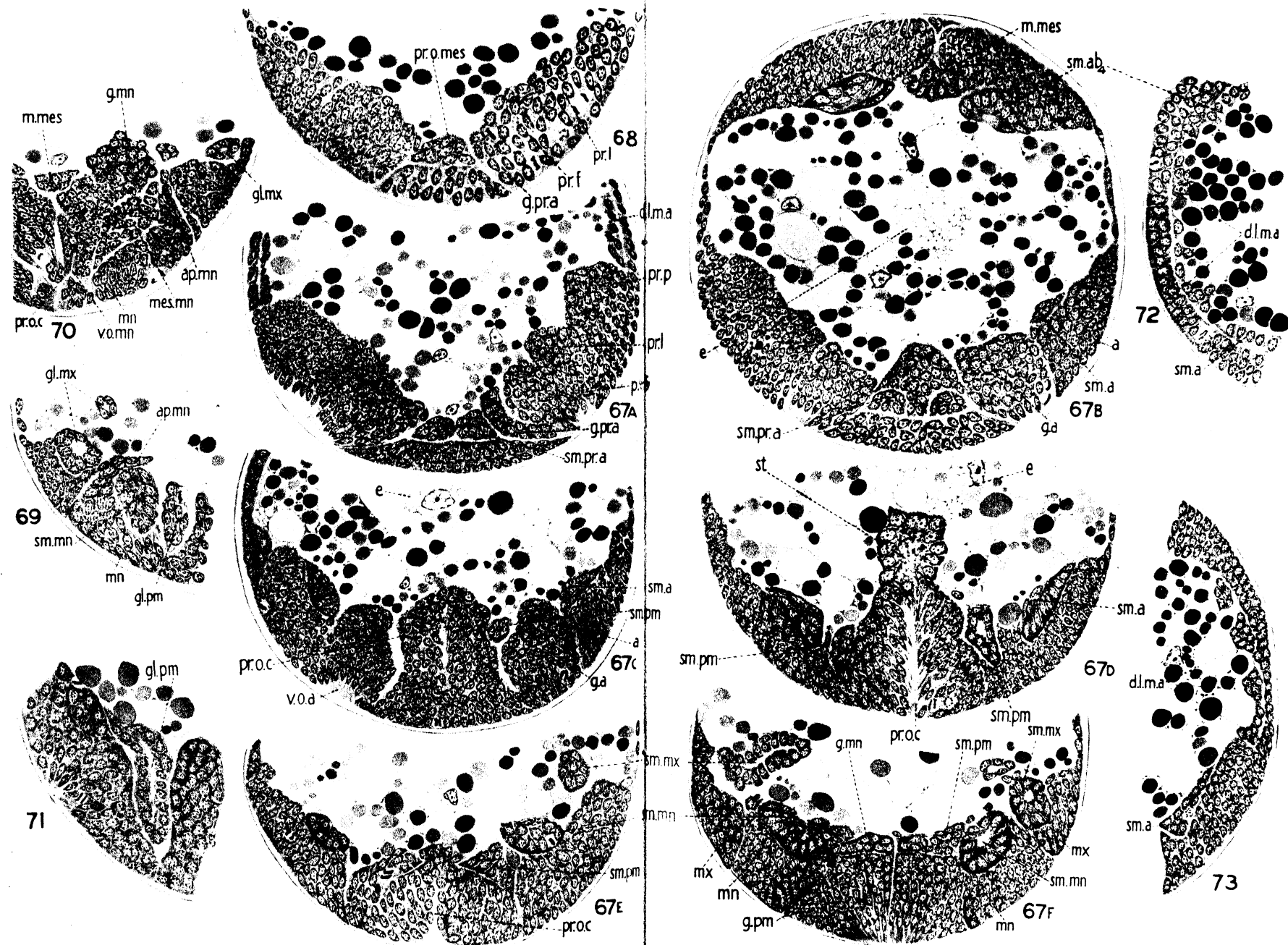
the height of their development, and also show mitosis. Neuropilem has begun to form in the ganglion. Note the inclusion of 'median mesoderm' in the ganglion. × 950.

Fig. 79. Portion of a section of a 7-day embryo, cut approximately, but not accurately, in the sagittal plane (the embryo is at about the same stage of development as that shown in Text-fig. 5). The section grazes along the lateral wall of the stomodaeum, and shows in succession the pre-mandibular, mandibular, maxillary, collum, and second abdominal somites. × 950.

Fig. 80. Similar section; anterior end to left. The section is from an 8-day embryo. Note that the maxillary somite has begun to elongate to form the salivary gland. The full length of the stomodaeum, which in this embryo has reached the central endoderm, is shown (from an adjacent section) by dotted lines. × 950.

Fig. 81. Similar section of a rather later embryo, anterior end to right. Stomodaeum not present in section. The section shows a more complete set of abdominal somites than does Fig. 80. They are now fully developed, and show each a minute coelomic cavity. Such a cavity is present also in the mandibular somite, but not in that of the collum segment. From the lower end of the maxillary gland a large clump of cells, the future maxillary myoblasts, is in process of separating away. × 950.

Fig. 82. Hinder end of a longitudinal section of a 9-day embryo, showing somites of the anal segment and fifth and fourth abdominal segments. × 950.

Fig. 83. Transverse section through an 8-day embryo, taken a little in front of the proctodaeal opening, the proctodaeum being therefore itself included in the section. In the large accumulation of pre-anal mesoderm no differentiation into somites has yet begun. × 950.

Fig. 84. Similar section, from a late 8-day embryo, showing initial phase in development of fifth abdominal somites. The single primordial germ-cell has now appeared. × 950.

Fig. 85. Similar section, from a 9-day embryo. On the left side a fully formed fifth abdominal somite is now present. × 950.

Fig. 86. Similar section, from an advanced embryo, showing disruption of fifth abdominal somite. The third pair of legs intrude, from in front, into the section. The 'ventral organs' of the fifth abdominal ganglion have appeared. × 950.

Fig. 87. Section through hinder end of a pupa, showing rudiment of the teloblastic mesoderm, from which the mesoderm of the new larval segments will develop. × 950.

Fig. 88. Section similar to that shown in Fig. 64, from an 8-day embryo. The maxillary somite is in process of conversion into the salivary gland. × 950.

Fig. 89. Portion of a section through the collum segment of a 10-day embryo. The midgut is in process of formation; on its roof the endodermal cells, with mesodermal investment, are beginning to form a regular epithelium, but its incomplete floor is still composed of large, irregular, and very reticulate 'yolk-cells'. The remainder of the 'yolk-cells' constitute the developing fat-body. The hinder end of the protocerebral ganglion intrudes into the section. The collum ganglion is still associated with its 'ventral organ'. Neuropilem is appearing; note the development of commissural fibres above the 'median mesoderm'. Part of the maxillary gland, including its hinder tip, intrudes into the section; note the developing 'end-sac'. The clump of myoblasts that has arisen by disruption of the collum somite is seen to the right of the ganglion. The two small epidermal cell-aggregations to the sides of the 'ventral organ' are tendon cells for attachment of some dorso-ventral muscles. × 950.

Fig. 90. Section through roof of pre-oral cavity, showing, on left, the earliest detected stage in formation of the clypeal gland. On the right side the gland has not yet begun to form. × 950.

Fig. 91. Portion of a section which grazes along the floor of the clypeus; 10-day embryo. The section actually cuts, below, a little into the pre-oral cavity. Note the developing clypeal glands, growing backward toward the frontal ganglion. × 950.

PLATE 8

Fig. 92. Left half of a 'horizontal' section taken at level of pre-oral cavity, from a 9-day embryo. Note that the 'ventral organs' of the developing pre-mandibular segment have moved up to the pre-oral cavity, and are now forming its lateral walls. A fragment of the enlarging pre-mandibular somite intrudes into the section. × 950.

Fig. 93. Part of a section through head of an 8-day embryo (from a 'horizontally' cut series). The section passes immediately in front of the developing pre-oral cavity, and is to be

compared with that shown in Fig. 67 A, which is from a slightly earlier embryo. The small 'ventral organ' of the pre-antennary ganglion is now at its maximum development. A 'ventral organ' cell disposition also involves the whole free surface of the lateral and frontal proto-cerebral lobes. × 950.

Fig. 94 A–E. Drawing of five approximately 'horizontal' sections through the head of a 10-day embryo. In Fig. B the entire section through the embryo has been drawn, to show (above) the transected abdomen. The series represents an incomplete succession of sections, two having been omitted between B and C, and one between D and E. The series should be com-pared with Fig. 67 A–F. × 950.

In A are seen the posterior lobes of the protocerebral ganglion, which have grown down from the roof of the head, and have almost met in the mid-line. To the side of these are the lateral lobes of the protocerebrum, of which that on the right side shows 'ventral organ' cell disposition. Anterior to these are the frontal lobes, which have fused in the mid-line; the left shows a 'ventral organ'. On the right side is seen the pre-antennary ganglion. Neuropilem is forming between the three component lobes of the protocerebrum.

B. This section lies immediately below the former. It is a little dorsal to the oesophagus, grazing, at one place, along a fragment of its roof. The lowest part of the posterior proto-cerebral lobe is present in the section. The deutocerebrum, with neuropilem, is forming, by fusion of the antennary ganglia above the oesophagus. The deutocerebrum therefore lies just below the pre-antennary ganglia, of which a fragment still intrudes from above into the section (right side). The upper half of the section shows the transected abdomen, at the level of the single primordial germ-cell. Note developing trichobothrium.

C. This section passes along the floor of the pre-oral cavity, the latter being transected at one point. The section includes the developing tritocerebrum; note its inferior commissure passing below the oesophagus; note also its 'ventral organs' which are becoming withdrawn from the postero-lateral wall of the pre-oral cavity.

D. This section lies immediately below C, and is taken at the level of the mandibular seg-ment. Note the mandibular ganglion, with its 'ventral organs' forming the floor of the pre-oral cavity. On the right side is seen the developing right hypopharyngeal apophysis, curving back round the ganglion. On both sides may be seen the deep ingrowths of the bases of the mandibles (mandibular apodemes) into the head.

E. This section lies a little distance below D (it should be compared with Fig. 98, which is from a rather earlier embryo). The section passes 'horizontally' along the floor of the maxillary segment, and shows the Anlage of the intermaxillary gland, now separated from the maxillary ganglion. The maxillary 'ventral organ' forms the tip of the intermaxillary plate.

Fig. 95. Section through the collum ganglion of a 10-day embryo, showing its 'ventral organs'. × 950.

Fig. 96. Equivalent section, from a more advanced embryo; a portion of the 'ventral organ' has remained within the sternal epidermis, but the cell-orientation has been lost. From this will develop the 'exsertile vesicle'. Although from a later embryo than the foregoing, the neurilemma has not yet appeared. × 950.

Fig. 97. Similar section, from a pupa, showing developing exsertile vesicle. × 950.

Fig. 98. Section through floor of maxillary segment of a 10-day embryo, to show Anlagen of intermaxillary glands, and beginning of separation of the latter from the maxillary ganglion. Two differentiating neurilemmal cells are seen above the neuropilem. × 950.

Fig. 99. Part of a section of an advanced embryo, from a sagittal series, the section being taken just to the side of the oesophagus. It shows the tritocerebral (pre-mandibular) ganglion, which lies to the side of the oesophagus, also the mandibular, maxillary, and collum ganglia. Note the developing intermaxillary gland lying below the maxillary ganglion. × 950.

Fig. 100. Fragment of a section through lateral head-wall of an advanced embryo, showing development of pseudocular gland (for orientation of section see Fig. 115 A). × 950.

Fig. 101. Drawing of an oblique section through the head of a 10-day embryo. The section is so directed that it passes, on the right side, through the frontal lobe of the protocerebrum, and on the left through the frontal and lateral lobes. The sagittal plane of the embryo, defined by the oesophagus and the septum (s) of epidermal cells, is therefore displaced to the right in the section. The obliquity of the section at once brings out the relation of the pre-antennary ganglion to the frontal lobe of the protocerebrum (right side), and to the antennary ganglion and neuropilem of the brain (left side). The relevant sections for comparison are Figs. 93, 94 A. × 950.

PLATE 9

Fig. 102. Frontal section through tip of head of a very advanced embryo, to show developing intermaxillary glands. On the roof of the head the section passes (on left) through rear of base of antenna, and shows the developing basal antennary sense organ. Included in the section are the frontal (visceral) ganglion, and part of the clypeal glands. × 780.

Fig. 103. Approximately sagittal section of a 9-day embryo, rather less developed than that shown in Text-fig. 8. The embryo shows the mid-gut in course of acquiring its mesodermal investment, the mesoderm spreading backward over it from the fore-gut. Four endoderm cells are shown in the section, and there is present also a large degenerate yolk-laden cell within the lumen of the developing mid-gut. The pre-antennary somite has disrupted into a clump of cells occupying the cavity of the clypeus. Behind the fourth abdominal segment the mesoderm has not yet differentiated into somites. The unsegmented median mesoderm is exceptionally clearly seen. The developing ganglia are massive and have begun to merge into a continuous chain. The pre-mandibular ganglion lies mostly to the side of the pre-oral cavity, and is therefore not seen; the mandibular ganglion has been drawn up near the hinder margin of the pre-oral cavity. Three 'ventral organs' are present in the section. The 'dorsal organ' is at the height of its development, and its secretion spreads outwards for a short distance under the blastodermic cuticle. × 780.

Fig. 104 A, B. Photographs of two parasagittal sections of a 9-10-day embryo. The photograph has been taken to show, as objectively as possible, the appearance of the 'ventral organs'. The 'ventral organs' seen in Fig. A are those of the collum and second and third abdominal segments. Fig. B, from the adjacent section, shows the maxillary 'ventral organ', and grazes also through the side of the mandibular 'ventral organ'. Other structures seen in the photograph are the 'median mesoderm', with aligned cells, a transected Malpighian tube, and a fragment of the fifth abdominal somite. Note also mid-gut lumen. × 600.

Photographs by Professor E. J. Hartung.

Fig. 105. Portion of a parasagittal section along floor of germ-band of an 8-day embryo, showing early stage in development of the ganglia. The section should be compared with the rather more advanced one shown in Fig. 103. The ganglion-rudiments are not yet continuous; they comprise, from left to right, the ganglia of the maxillary, collum, and first two leg-bearing segments. The 'ventral organs' of the first two only are present in the section. × 780.

Fig. 106. Part of an approximately transverse section through a 10-day embryo; the section passes (below) through the base of the second leg, and (above) through the tergal wall of the fifth abdominal segment, whose developing trichobothrium may be seen. The anterior tip of the hind-gut is also transected, and one of the Malpighian tubes is seen in process of growing out from it. Note the 'ventral organs' in process of incorporation into the ganglia. In the adjacent mesoderm the Anlage of the ventral longitudinal muscle is distinguishable; note also masses of myoblasts within the leg, while others are spreading up the lateral body-wall. × 780.

Fig. 107. Approximately transverse section through third abdominal segment of an advanced embryo. Fragments of the second pair of legs are present in the section. The dorsal body-wall has formed. There is now a well-developed mid-gut, showing marked contrast between the irregularly reticulate cells on its floor and the more compact epithelium on its roof. In the latter concretions have appeared. The 'yolk-cells' are now recognizable as developing fat-body. The nerve-cord has completely separated from the epidermis, and the 'ventral organ' cell disposition has disappeared. The developing ventral longitudinal muscles are now very conspicuous. On the right side is seen a developing coxal apodeme. On the left is seen, in course of development, one of the large muscles of the base of the leg. × 780.

Fig. 108. Similar section to foregoing, from a pupa. There is no further advance in development of the mid-gut. The fat-body has undergone a further depletion of its yolk. It has begun to shrink from the neighbourhood of the nerve-cord, thereby revealing the epineural and lateral neural blood-spaces. The ventral longitudinal muscles have moved into their definitive positions to the side of the nerve-cord. × 780.

Fig. 109. Similar section, from a very young first instar larva. The development of the mid-gut wall is now complete, and it is wholly enclosed by mesoderm. A 'striated border' has formed in the mid-gut epithelium, but is absent on the floor-cells. The fat-body is completely denuded of its yolk, and reserve products have not yet begun to accumulate, the blood-spaces being therefore wide. × 780.

Fig. 110. Frontal section through head of a 9-day embryo. The section passes along the length of the antenna, 'horizontally' through the floor of the mandibular segment, and grazes

the maxillary segment below. The antenna is occupied by a clump of cells, derived from the disrupting antennary somite; some of its cells are extending back, under the roof of the head, to form the tergal muscle of the antenna. Median to the antenna is the antennary ganglion (developing deutocerebrum), and below it a fragment of pre-mandibular ganglion (developing tritocerebrum). The ectoderm along the lateral margins of the mandible and maxilla is growing into the head to form their respective apodemes, which are drawing in some of the mesoderm with them. × 780.

Fig. 111. Frontal section through right half of head of an embryo aged about 10 days. The plane of section is so directed that the pre-mandibular gland is included for its entire length in the section. Below it lies the mandible, now deeply ingrown into the cavity of the head, and displaying at its inner end the developing lateral mandibular ligament. Below the mandible the section grazes the surface of the maxilla. In the epidermis some unusually large cells are to be seen; those above the brain are setigerous cells, those to the right of the brain are the enlarging cells of the pseudocular gland. In the brain we can distinguish the two diminutive pre-antennary ganglia, between which the septum of ectodermal cells is growing down from above. To the side of the pre-antennary ganglion, the great mass of ganglionic tissue is the protocerebrum, of which the frontal and lateral lobes are distinguishable. Below the pre-antennary ganglion is seen a portion of the developing deutocerebrum, the antennary ganglia having begun to fuse above the oesophagus. A portion of the deutocerebrum also intervenes between the protocerebrum and the pre-mandibular ganglion (developing tritocerebrum), which may be seen to the side of the oesophagus. × 780.

Fig. 112. Portion of a frontal section through an advanced embryo, showing the cells of the hypopharyngeal apophysis becoming connected with the overlying antennary and the underlying mandibular mesoderm. For an earlier stage of development, see Fig. 115 A.

Fig. 113. 'Horizontal' section along hind-gut of an advanced embryo, showing incipient differentiation of colon and rectum. Note the incorporation of some hind-gut cells into the wall of the mid-gut. The Malpighian tubes are beginning to develop. × 780.

Fig. 114. 'Horizontal' section of mid-gut of a young pupa; the ends of the oesophagus and hind-gut are included in the section, and, on the right side, a Malpighian tube (base of other Malpighian tube on left). Differentiation of the Malpighian tube into its three regions has already begun. Note excretory concretions in mid-gut cells. × 780.

Fig. 115 A, B. A is a drawing of a frontal section through the anterior end of a 10-day embryo. The section passes (above) through the protocerebrum, grazes along the hinder surface of the deutocerebrum and tritocerebrum, and transects (below) the 'ventral organs' of the maxillary segment. The ganglion above the 'ventral organ' is not purely maxillary ganglion: it is the sub-oesophageal ganglion, of which the lower part in contact with the 'ventral organs' is of maxillary, the upper of mandibular, origin. The mandibular ventral organs, of course, lie anterior to the plane of section (for orientation of section refer to Text-fig. 8). To the sides of the sub-oesophageal ganglion are seen the transected inner ends of the mandibular apodemes, from which a few cells are beginning to grow medially across the mandibular ganglion; they give the first indication of the oesophageal (visceral) ganglia. The small clump of cells between the mandibular apodeme and the fragment of tritocerebrum is the hindermost tip of the hypopharyngeal apophysis. A little antennary mesoderm intrudes from in front into the section, a fragment of the antenna being also present. In the lowest part of the section is seen a little maxillary mesoderm. To the side of the protocerebrum the development of the pseudocular gland is in progress.

B is a fragment of the immediately succeeding section of the same embryo. The section shows the maxillary apodeme and the mesoderm of the segment, growing up the side of the ganglion, towards the hypopharyngeal apophysis, whose hinder end is seen in the preceding section. × 780.

The following abbreviations are used in the lettering of the Plates:

a antenna; *a.o* anal opening; *ap.cx* coxal apodeme; *a.pl* anal plate; *ap.mn* mandibular apodeme; *ap.mx* maxillary apodeme; *b.a.s.o* basal antennal sense organ; *b.c* blastodermic cuticle; *b.d.m* buccal dilator muscle; *br* brain; *c* colon; *cl.f* clypeal fold; *c.pr* protocerebral commissure; *c.tr.i* inferior tritocerebral commissure; *dev.sm* developing somite; *d.l.m* dorsal longitudinal muscle; *d.l.m.a* Anlage of dorsal longitudinal muscle; *d.mx* exit duct of maxillary gland; *d.o* 'dorsal organ'; *d.pm* exit duct of pre-mandibular gland; *dt* deutocerebrum; *d.y.c* degenerating yolk-cells; *e* endoderm; *e.d* developing exit duct of reproductive organs; *e.d.a* anterior part of ejaculatory duct; *e.d.deg* degenerating exit duct of female reproductive system; *e.d.g* glandular

part of ejaculatory duct; *ep* epidermal cells; *ep.s* epineural sinus; *e.s* 'end sac' of salivary gland; *ex.v* exsertile vesicle (?); *f.b* fat-body; *f.b.s* developing secondary fat-body; *f.c* follicle cell; *f.p.n* female pro-nucleus; *g.a* antennary ganglion; *g.ab₂, ₃*, &c. ganglia of second, third, &c. abdominal segments; *g.c* ganglion of collum segment; *g.f* frontal (visceral) ganglion; *gl* globulus; *gl.cl* clypeal gland; *gl.i.mx* intermaxillary gland; *gl.mx* maxillary (salivary) gland; *gl.pm* pre-mandibular gland; *gl.ps* pseudocular gland; *gm* germarium; *gm.c* germ-cells; *g.mn* mandibular ganglion; *g.mx* maxillary ganglion; *gn.a* genital atrium; *gn.t* genital tube (as distinct from its content of germ-cells); *gn.t.a* Anlage of genital tube; *g.oes* oesophageal ganglion (?); *g.oes.a* its Anlage; *g.pm* pre-mandibular ganglion; *g.pr.a* pre-antennary ganglion; *g.sb.oes* sub-oesophageal ganglion; *g.tel* teloblastic ganglion; *h.a* hypopharyngeal apophysis; *h.a.a* ascending arm of latter; *h.g* hind-gut; *h.l* head-lobe; *i.mx.pl* intermaxillary plate; *l₁, ₂*, &c. first, second, &c. legs; *l₅a* Anlage of fifth leg; *l.m.h* levator muscle of head; *l.n.s* lateral neural sinus; *mes* mesoderm; *mes.mn* mandibular mesoderm; *mes.mx* maxillary mesoderm; *mes.s.a* sternal mesoderm of antenna; *m.g* mid-gut; *m.g.mes* mid-gut mesoderm; *m.g.p* proctodaeal component of mid-gut; *m.mes* 'median mesoderm'; *mn* mandible; *mn.l.l* lateral 'ligament' of mandible; *mn.l.m* median ligament of mandible; *mn.st* mandibular sternite; *m.p.n* male pro-nucleus; *m.t* Malpighian tube; *m.terg.a* tergal muscle of antenna (Anlage of); *mx* maxilla; *my.ab₂, ₃*, &c. myoblasts of second, third, &c., abdominal segments; *my.c* myoblasts of collum segment; *my.mx* myoblasts of maxillary segment; *n* neuropilem; *ng* neuroglia cells; *nl* neurilemma; *o* stomodaeal opening; *oc* oocyte; *od* oviduct; *oes* oesophagus; *oes.d.m* oesophageal dilator muscle; *og* oogonia; *o.s* occipital suture; *ov* ovary; *o.w* ovarian wall; *p* penis; *p.b* polar body; *pr* proctodaeum; *pr.f* frontal lobe of protocerebrum; *pr.g.c* primordial germ-cell; *pr.l* lateral lobe of protocerebrum; *pr.mes* proctodaeal mesoderm; *pr.o.c* pre-oral cavity; *pr.o.mes* pre-oral mesoderm; *pr.p* posterior lobe of protocerebral ganglion; *prt* protocerebrum; *p.s* pupal sheath; *r* rectum; *r.s* receptaculum seminis; *r.s.a* Anlage of latter; *r.v* rectal valve; *s* septum of epidermal cell● growing down between pre-antennary ganglia; *s.a* anal segment; *s.ab₂, ₃*, &c. second, third, &c., abdominal segments; *s.c* collum segment; *sm.a* antennary somite; *sm.ab₂, ₃*, &c. second, third, &c., abdominal somites; *sm.an* anal somite; *sm.c* somite of collum segment; *sm.mn* mandibular somite; *sm.mx* maxillary somite; *sm.pm* pre-mandibular somite; *sm.pr.a* pre-antennary somite; *s.pm* pre-mandibular segment; *s.s₂, ₃* second, third sensory setae (trichobothria); *s.s.a* Anlage of sensory seta (trichobothrium); the particular seta may be identified by a numeral, e.g. *s.s.₂a*, &c.; *st* stomodaeum; *st.c* setigerous cell; *st.m* musculature of stomodaeum; *st.mes* stomodaeal mesoderm; *sus* suspensorium of hypopharyngeal apophysis; *t* testis; *tel.mes* teloblastic mesoderm; *tn* 'tendon' cells, for attachment of muscles; *tr* tritocerebrum; *undif.mes* undifferentiated mesoderm; *v.d* vas deferens; *v.l.m* ventral longitudinal muscle (segmental allocation of this muscle sometimes indicated by numerals); *v.l.m.a* its Anlage (segmental allocation sometimes indicated by numeral); *v.o.a, v.o.ab₂, ₃*, &c., *v.o.c, v.o.mn, v.o.m.x, v.o.pm* 'ventral organs' of antennary, second and third, &c., abdominal, collum, mandibular, maxillary, and pre-mandibular segments respectively; *v.o.tel* 'ventral organ' associated with teloblastic ganglion; *v.s* vesicula seminalis; *v.s.a* its Anlage; *x, y* for various uses of these letters see legend to figures in which they appear; *y.c* yolk-cells; *y.n* yolk nucleus; *z* as for *x*.

Some Observations with the Phase-contrast Microscope on the Neurones of *Helix aspersa*

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With 1 Text-figure

ALTHOUGH various investigations have contributed to the development of phase-contrast microscopy from the time of Abbe, the production of a completed microscope available for use in biological and allied fields has been a comparatively recent achievement (Köhler and Loos, 1941). A good review of this promising advance is given by Bennett (1946).

The neurones of *Helix aspersa* have been chosen for the present study as these cells are large and easily isolated in a fresh state, as well as for their having already been the subject of a number of cytological investigations (Kolatchev, 1916; Brambell, 1923; Brambell and Gatenby, 1923; Boyle, 1937). The following observations are limited to a description of the appearance of the neurofibrils in these cells together with a description of some very small particles shown to be present in their protoplasm, distinct from and of a much smaller order than the mitochondria. A further communication is on hand concerning some observations on the other inclusions and organoids of these cells.

TECHNIQUE

The large motor neurones of the post-cerebrum of *Helix* were dissected, with the aid of a Greenough microscope, in a few drops of 0·7 per cent. sodium chloride containing 0·2 per cent. of 10 per cent. anhydrous calcium chloride. The fluffy nerve-cell mass can be detached from the firm connective tissue capsule of the cerebral ganglion and subsequently teased and flattened in a drop of the indifferent solution between cover-glass and slide. In this way it is possible to display a single-cell layer of the large neurones arranged in a fan-shaped manner, their unipolar cell bodies and stout axons resembling a group of captive toy balloons. The degree of flattening of the cells due to the cover-glass pressure makes possible high-power observations on their cytoplasm with the phase-contrast microscope. Similar preparations examined in hanging drops were found to be suitable only for low- and medium-power observation, as the full benefit of phase-contrast illumination can apparently only be obtained in preparations consisting of a single layer of cells.

The microscope used was kindly lent me by Dr. W. Loos and was supplied with an annular illuminant, pankratic condenser, and an annular diffraction
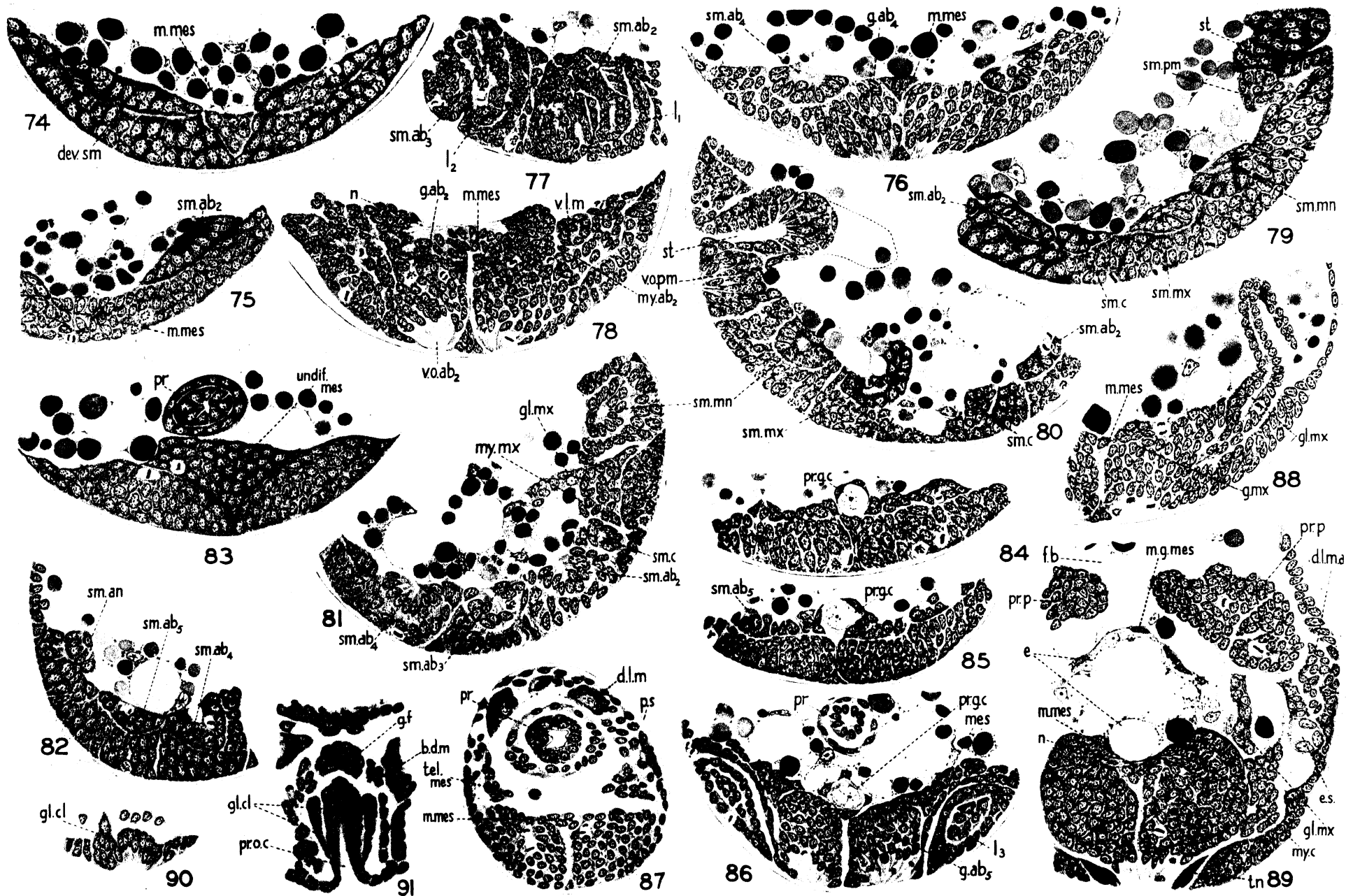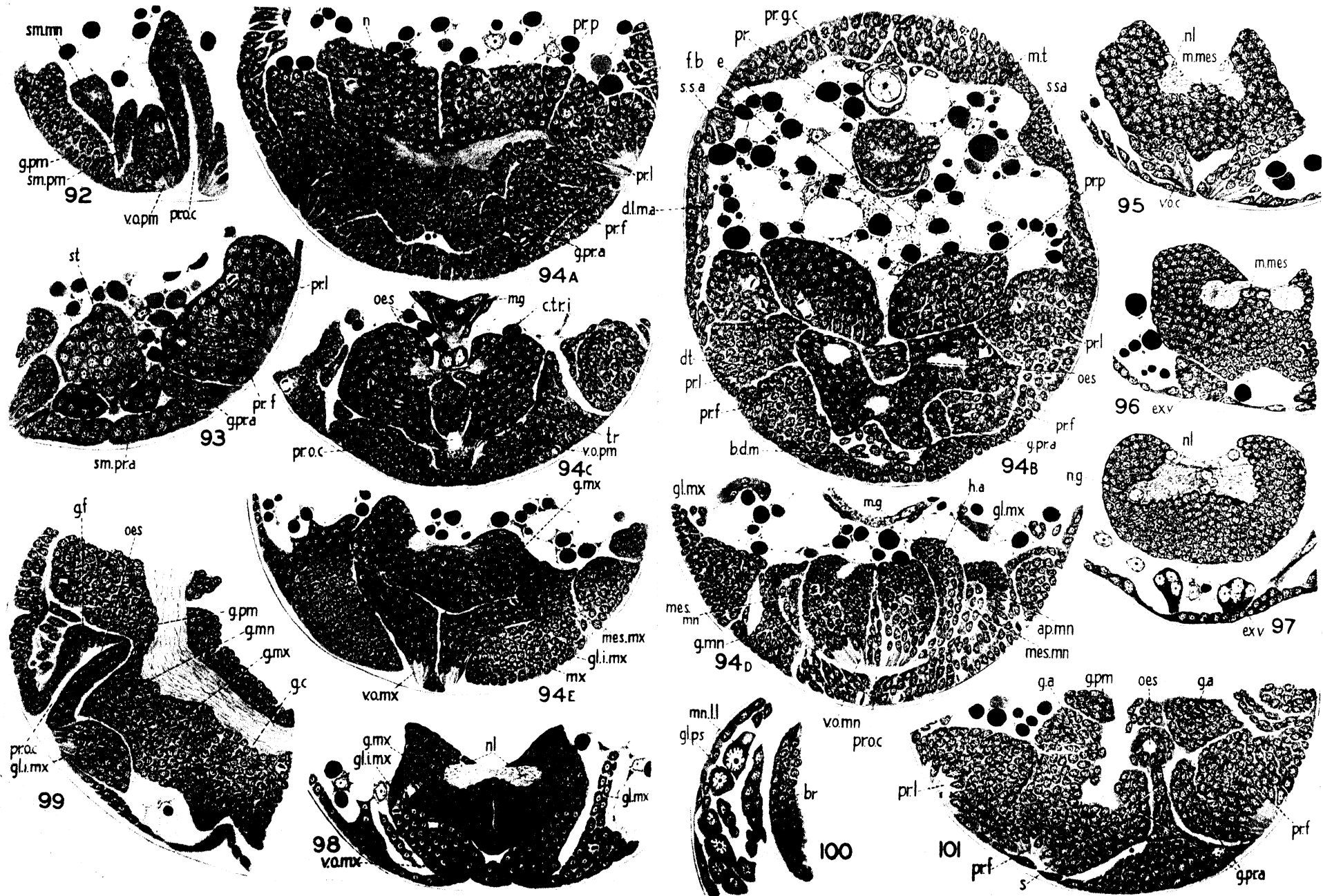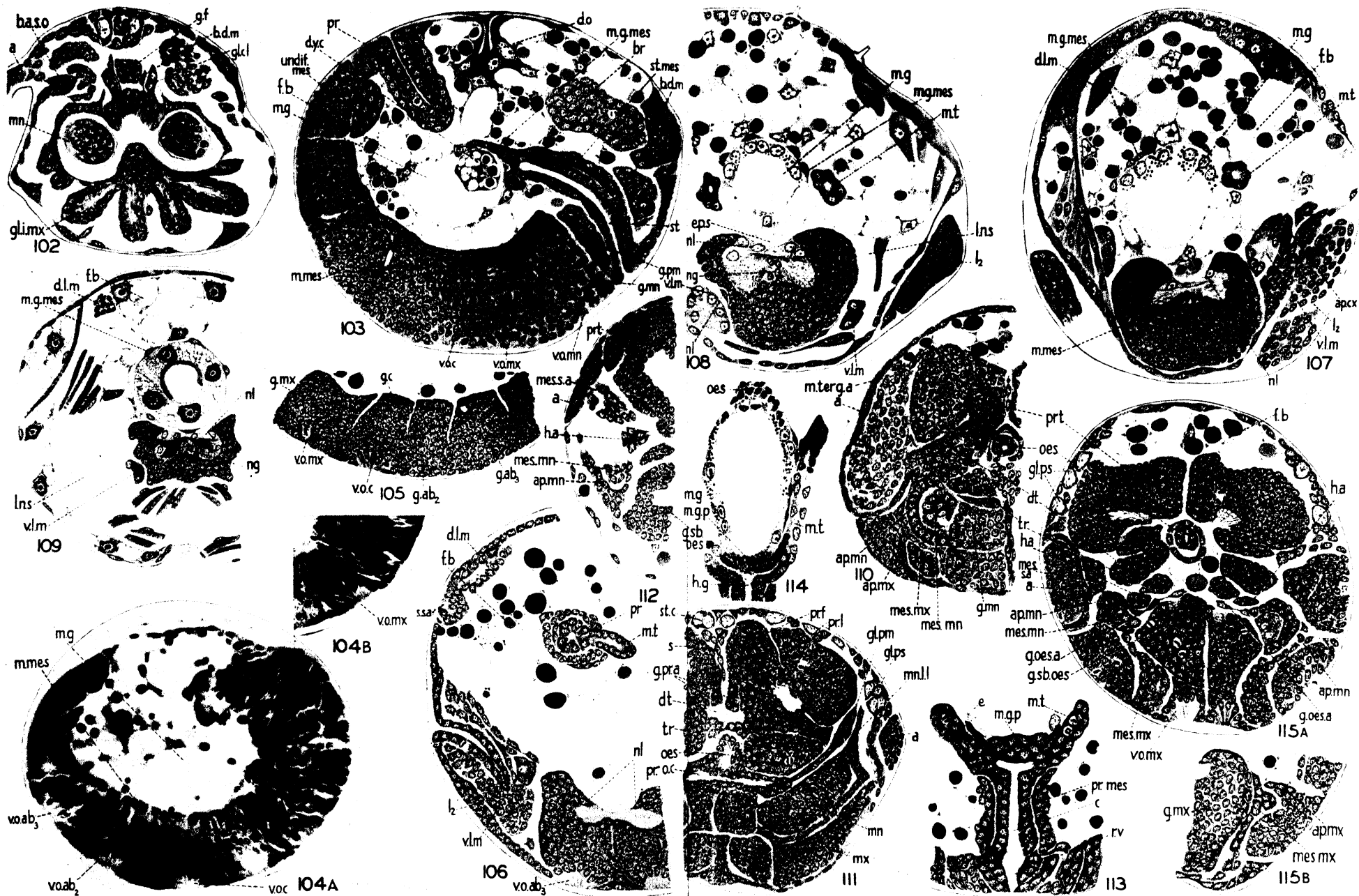
plate of the A+ type of Bennett (1946) incorporated within the optical system of an achromatic 1·25 n.a. homogeneous immersion lens. This was used in conjunction with a 10× compensating ocular.

OBSERVATIONS

(a) The Neurofibrils

The large unipolar neurones exhibit a groundwork of very fine filaments which are arranged in rows parallel to the axon. Each filament is delicately beaded in structure and resembles a very long chain of minute cocci. As the axon hillock is reached the rows of fibrils fan out and form a continuous
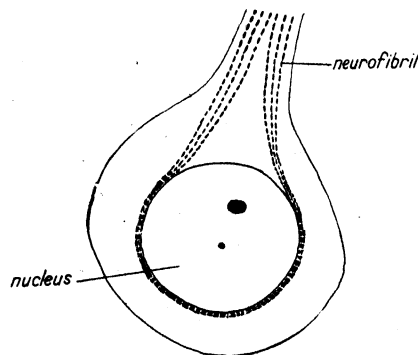
TEXT-FIG. 1. Schematic drawing of the arrangement of the neurofibrils in a large neurone of *Helix* as seen in optical section with the phase-contrast microscope.

filamentous sheath enveloping the nucleus, the individual fibrils passing in an uninterrupted fashion around the nucleus and back into the axon. No evidence of lateral anastomoses of these filaments either within the axon or in the cell body was observed.

With ordinary transmitted illumination they cannot with certainty be recognized; but if a quick transition from transmitted to phase-contrast illumination be made on the same field, the impression of a very faint striation at once gives place to the well-defined picture described above. This experiment is itself a very striking one and shows to full advantage the superiority of the new method of illumination for the study of these structures.

As a 'control' to these observations *in vivo*, preparations were made with the classical reduced silver technique of Cajal as modified by Nonidez (1939) and the presence of neurofibrils in *Helix* neurones was established by this method. In this way their size and general distribution within the cell conforms to the appearances seen with the phase-contrast microscope, but the beaded appearance of the fresh neurofibrils was not reproduced in the fixed and silvered tissue. Moreover, their regular and orderly arrangement in the fresh cell was severely distorted by the fixation and silvering process. This was especially noticeable about the nucleus, where the neurofibrils appear to form a coarse network of anastomosing threads.

(b) The Microneurosomes

When preparations of the teased cells compressed between cover-glass and slide are watched for a few minutes, spherical blebs of cytoplasm are seen to exude from the cell membrane of the neurones and finally to detach themselves and float away in the surrounding medium. These droplets usually appear as clear homogeneous filtrates of the cell cytoplasm when ordinary transmitted illumination is employed in their examination. Occasionally a few cell granules such as mitochondria may escape into the droplet from the parent cell.

If the phase-contrast microscope is turned to their examination the impression of this clear homogeneity is immediately lost and they are found to be teeming with an immense number of very small particles moving rapidly with an intense Brownian motion. The individual particles can be distinctly resolved by the eye but it is impossible to see their exact shape and it is impossible to measure them by any of the means ordinarily at the disposal of the microscopist.

After a few minutes of very active movement the tiny particles slowly commence to agglutinate and finally the whole droplet becomes a motionless pale-grey mass of cytoplasm. It was found that 10 per cent. formol or 70 per cent. alcohol run under the cover-glass by diffusion produced an instantaneous fixation and agglutination of the particles. Furthermore they are conveniently made to adhere either to the slide or cover-glass and the preparation can then be stained and mounted in balsam. Toluidine blue and methylene blue were both used as staining agents and strongly stain the agglutinate.

Attempts were made to stain these particles with vital dyes, and neutral red 1 : 10,000 in saline was found to give the best results. In this way the tiny particles are quickly tinged a brick-red colour and although their small size precludes accurate description as to shape, the individual particles are distinctly coloured. Similarly the agglutinated mass retains the red colour. When stained vitally the particles are visible with transmitted light provided a well-corrected immersion lens be used with a wide cone and 3 mm. diaphragmed source of light. These particles are not to be confused with the vacuome of Parat, which in these cells consists of much larger red-staining spheres.

It must be stressed that these particles are only seen to perfection once they have been expressed from the cell. With careful searching similar particles can be seen to occur within the intact cell, especially over the nucleus where the cytoplasmic layer is naturally reduced in thickness. Boyle (1938) states that he never witnessed Brownian movement within *Helix* neurones, using this argument to support his contention that the cytoplasm of the cell in life is of semi-solid consistency. I am not in agreement with this finding.

DISCUSSION

The status of the neurofibrils as structural components of the living nerve-cell has long been a bone of contention among neurologists. Since their first reported discovery by Remak (1843) in the freshly teased nerve-cells of the

crayfish, all manner of opinion has been expressed from time to time concerning the reality of what Cajal has called the 'enigmatic warp' (Marinesco, 1911; Mott, 1912; Bozler, 1927; de Renyi, 1932). Claims for their existence in the neurones of a wide variety of both vertebrates and invertebrates have always been answered by counterclaimants who attribute their appearances as artifacts produced by the manipulative procedures of technique.

By virtue of its construction the phase-contrast microscope allows us to visualize very slight variations of optical density within what would appear as an optically homogeneous medium. Attention is drawn in this paper to the fact that structures apparently identical with the classical neurofibrils can be beautifully shown up by applying this principle of microscopy to teased cells lying in indifferent media. The only element of manipulative procedure inflicted upon the cells is the pressure of the cover-glass found necessary to flatten them adequately for high-power observation.

The very small particles appear to be structures peculiar to neurones. A large number of other tissues from the snail as well as from vertebrates have been examined with a similar technique, so far with negative results. In size and general conformity the particles appear to be of the order of phage-particles recently seen with the phase-contrast microscope (Hofer and Richards, 1945).

Although the possibilities of phase-contrast microscopy do not appear to have been fully developed, the instrument in its present form does not effectively increase resolution as such, nor does it allow us to visualize colloid particles in the manner of the ultramicroscope.

I am quite certain that the particles I have described do not correspond to those already observed in vertebrate neurones by Mott (1912) in his studies with the paraboloid dark field condenser and the ultramicroscope. Mott describes his particles as being less than one micron in size and sometimes spherical and sometimes oval in shape. He considered that they consisted of a colloidal fluid surrounded by a membrane possibly consisting of lipoids. Particles answering this description do occur in my material and are quite distinct from the much smaller neutral red-staining granules. As these granules can hardly be classed with the submicroscopic particles of Claude (1943) and further in size and staining reactions can be differentiated from the granular mitochondria of the neurone (the neurosomes of Held), I propose the name 'microneurosomes' for their description.

In conclusion I wish to thank Dr. J. R. Baker for his help and encouragement throughout this study.

SUMMARY

1. Phase-contrast microscopy reveals the presence of neurofibrils within the freshly teased neurones of *Helix aspersa*.

2. Each fibril resembles a row of minute cocci. Individual fibrils appear to pass from the axon to the cell body, loop round the nucleus, and retaining their identity pass back into the axon. No evidence of lateral anastamoses nor network formation of the fibrils could be found.

3. Small granules, staining vitally with neutral red and designated 'micro-neurosomes', have been found in the cytoplasm of the neuron. They are distinct from and smaller than the mitochondria.

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The Development and Affinities of the Pauropoda, based on a Study of *Pauropus silvaticus*

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PART II

(Continued from *Quart. J. micr. Sci.*, vol. 88, part 2, p. 165 (1947))

With 2 Plates and 7 Text-figures

CONTENTS

OBSERVATIONS ON POST-EMBRYONIC DEVELOPMENT

THE following is not intended as an exhaustive description of the whole larval development, but is restricted to those aspects of it to which special significance attaches.

1. *External Characters of Larvae*

(*a*) *First Instar Larva*. A description of this has already been given (section 6, iv).

(*b*) *Second Instar Larva* (Text-fig. 23 A). Length about 0·4 mm.

On the antenna three basal segments are now present, the new segment having been generated from thickened epidermis at the base of the antenna, and not at the tip. In each flagellum the number of rings has increased.

In the abdomen only one additional segment is present, i.e. eight in all. As will be seen in section 2, the new segment is the seventh abdominal, which has become interpolated between the sixth and anal segments of the previous instar, whose fifth and sixth segments have enlarged and acquired each a pair of legs. The sixth segment, like the fourth, is wedge-shaped with reduced tergal wall, the third tergal shield being the product of the fifth
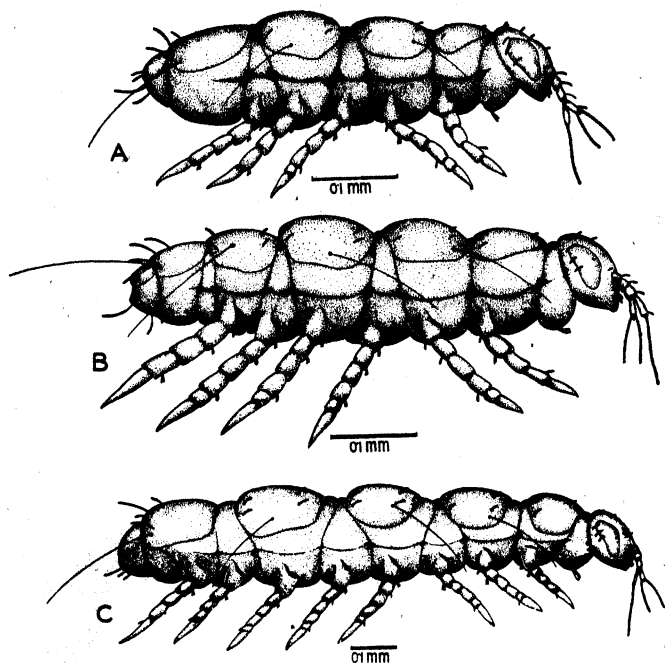
TEXT-FIG. 23. Larvae; A second, B third, C fourth instars.

segment alone. The new (seventh) segment is large and has a tergal shield bearing a pair of trichobothria.

The two newly formed legs are five-segmented, their tarsi being undivided. In the second and third legs, on the other hand, the hitherto unsegmented tarsi have divided into two segments. The first leg remains permanently with unsegmented tarsus. The legs progressively increase in length towards the hinder end of the abdomen.

(c) *Third Instar Larva* (Text-fig. 23 B). Length about o·5 mm.

In the abdomen there are ten segments, two additional segments having been acquired, viz. the eighth and ninth. The eighth segment, like the sixth and fourth, is a wedge-shaped segment, without a tergal shield. The ninth, like the seventh and fifth, is a large segment, with tergal shield, and it bears the new (fourth) trichobothrium. Neither segment bears legs in this instar.

On the seventh segment the sixth pair of legs has formed, its tarsi being unsegmented. The tarsi of the fourth and fifth legs have become bi-segmented.

(*d*) *Fourth Instar Larva* (Text-fig. 23 c). Length about 0·7–0·8 mm. Within the antenna the fourth (and last) basal segment has appeared.

In the abdomen there are eleven segments, one additional segment having been acquired, namely, the tenth. This segment, like the alternate segments that precede it, is a wedge-shaped segment, without a tergal shield, and remains permanently without one. It does not yet bear legs.

On the eighth and ninth segments, which were the new segments of the previous instar, the seventh and eighth legs have formed, their tarsi being unsegmented. The tarsi of the sixth legs have become bisegmented.

(*e*) *Adult Animal* (Text-fig. 24). Length 1–1·2 mm.

In the abdomen there are twelve segments, the newly acquired segment being the eleventh or permanent pre-anal segment. Like the alternate segments that precede it, it is a large segment, with a tergal shield (sixth of the series) and bears a pair of long trichobothria, but it is, and remains, devoid of legs. Its sternal wall is markedly reduced, and, like the anal segment, bears a well-sclerotized sternite.

On the wedge-shaped tenth segment, which was the new segment of the previous instar, a pair of legs has formed. Being the last of the series of enlarging legs, they are the most strongly developed of all; nevertheless they follow the general rule of first appearing with unsegmented tarsi, and since there is no further ecdysis, remain permanently in this state. The two preceding pairs of legs have now acquired bisegmented tarsi.

The fact that the last leg, though the largest of the series, should remain permanently in this incompletely differentiated condition, suggests that additional ecdyses may have been suppressed in *Pauropus*, and points even to the possibility that further teloblastic segmentation may have been arrested. This conclusion is supported by the discovery of *Decapauropus*, in which an additional segment (twelfth) is present between the anal and eleventh segments, the latter bearing an additional pair of legs (Remy, 1931).

2. *The Formation of New Segments and their Ganglia*

In the following account it will suffice to describe in detail the development of the teloblastic segments during the first larval stadium. According to Silvestri (1902) 'the formation of new segments takes place between the last segment of the trunk, which always remains small during the entire larval period, and the one preceding it'; opposed to this is Harrison's statement that the new segments appear between the last leg-bearing and 'pre-anal' segments. To examine the teloblastic growth of segments in the larva, it is necessary to look beneath the chitin, for a bare inspection of the external characters of successive larval instars does not suffice to reveal its nature.

The segmental muscles are a particularly useful guide, and they at once show that the fifth and sixth segments of the future second instar larva are the fifth and sixth of the first instar, which need mainly the legs to com-
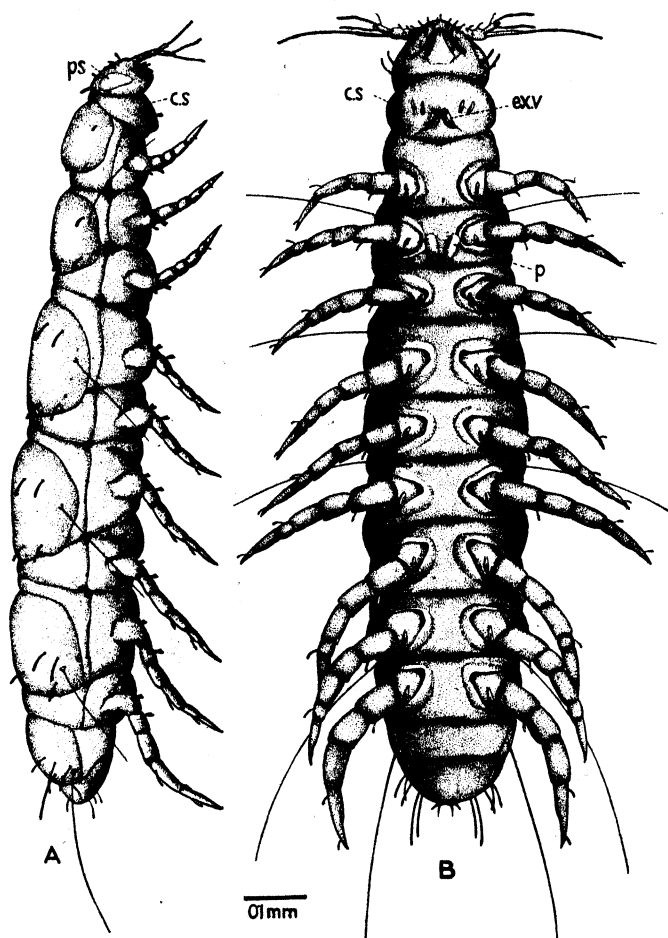
TEXT-FIG. 24. Adult.

A. Lateral view of a fully extended animal. B. Ventral view of a male.

Lettering. *c.s* collum segment; *ex.v* 'exsertile vesicle'; *p* penis; *ps* pseudoculus.

plete their development. The new segments, as Silvestri observed, must therefore develop in front of the anal segment, and the supposed stationary 'pre-anal segment' of Harrison is an error of observation.

The muscles of the first instar larva are shown in Text-fig. 25. By comparison with Text-fig. 22 C it will at once be seen that the tergal muscles

of the third leg, which in the adult are connected above to the second and third tergal shields, arise in the larva from the second and third shields respectively. Similarly, the second oblique muscle (*obl.b*) of the larva, which is attached above to the hinder end of the third shield, is attached in the adult also to the hind end of the third shield. It is clear, therefore, that the third shield of the first instar larva must become the third abdominal shield of the next and all later instars. That its sensory seta (trichobothrium) must become the second of the series is proved by the fact that when the fifth
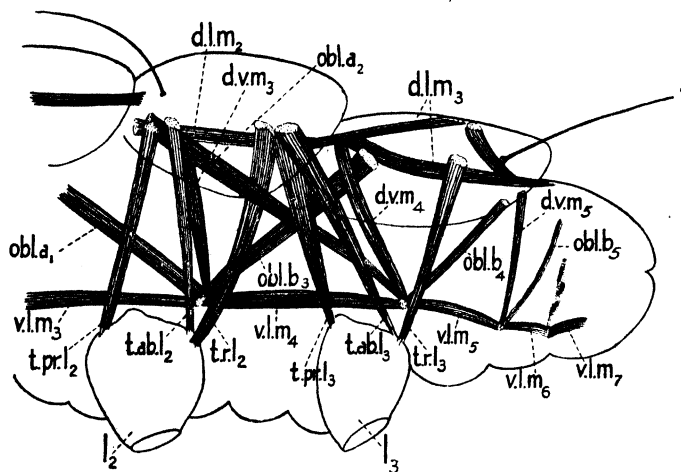
TEXT-FIG. 25. Hinder end of trunk of a first instar larva, drawn to show muscles. The drawing should be compared with Text-fig. 22 C, in which the same muscles are identified by attached numerals.

Lettering. d.l.m dorsal longitudinal muscle; *d.v.m* dorso-ventral muscle; $l_{2, 3}$ second and third legs; *obl.a, obl.b* two sets of oblique muscles; *t.ab.l* tergal abductor of leg; *t.pr.l* tergal promotor of leg; *t.r.l* tergal remotor of leg; *v.l.m* ventral longitudinal muscle.

abdominal ganglion becomes defined in the first instar larva, the nerve to the trichobothrium is found to arise from it, and not from the teloblastic ganglion. The clue to the sternal parts of the new segments is given by the ventral longitudinal muscles, those of the fifth and sixth segments being already present in the newly emerged first instar larva.

Turning now to the actual process of formation of new segments, it will be well to begin with the advanced embryo, in which the demarcation of the fifth abdominal segment from the anal takes place (cf. section 6, ii, A). In the 9-day embryo intersegmental lines appear in the abdomen, but behind the fourth abdominal segment the development of such lines is delayed, and it is not until a little before the pupa forms that an indication of the line between the fifth abdominal and anal segments is seen (fig. 31, Pl. III). Yet when the pupa is set free, there is already a deep groove in the epidermis between these segments (fig. 32 A, Pl. III). The larva, when this in turn

emerges, displays yet another new segment; it is the sixth abdominal segment, i.e. a wedge-shaped segment with reduced tergal wall, and is interpolated between the fifth and anal segments (fig. 34, Pl. III). How has this new segment arisen?

It may have arisen by division of the original fifth segment; or it may have developed out of cells which spread forward from a zone of proliferation in the anal segment. That the latter is the case is shown by examining the ventral longitudinal muscles. In the terminal segments the development of the muscles takes place much later than in the leg-bearing segments, the fifth muscle not appearing until about the time that the pupa is due to leave the egg. This muscle is attached behind, at the intersegmental line between the fifth and anal segments. It is readily identified in the first instar larva, where it is still the ventral longitudinal muscle of the fifth segment; the new (sixth) segment could therefore not have arisen by division of the original fifth segment, but must have been generated from behind. The process of teloblastic growth therefore actually begins in the pupa.

In the newly emerged larva the terminal segments present the following features (fig. 34, Pl. III; Text-fig. 26 A): within the anal segment the epidermal cells, though seldom more than one deep, lie closely crowded, this crowding of cells involving all its lateral walls, and the hinder part of the roof of the segment, together with most of the floor anterior to the line of attachment of the occlusor ani muscles. The attachment of these muscles behind the zone of proliferation ensures that they remain fixed as the proliferating epidermal cells spread forward. In the fifth and sixth segments the epidermis is for the most part thin; the floor of the segments is, however, greatly thickened, owing to the presence of the developing ganglia, while to the side there is also a thickening, though less pronounced, in the region of impending leg-formation (fig. 127, Pl. X). In these terminal segments there are still only two ganglia (Text-fig. 26 A), as in the advanced embryo and pupa (cf. Text-figs. 8, 9). Of these the more anterior is the fifth abdominal, now sharply delimited, but still in course of enlargement, for mitoses are often seen in its cells, and its 'ventral organs' are still present. With it is connected the nerve from the second trichobothrium. Behind the fifth ganglion, on the floor of the sixth segment, is the teloblastic ganglion, but the future sixth ganglion has not yet become demarcated from it. With the teloblastic ganglion is merged the vestigial anal ganglion, and from it arises also the caudal visceral ganglion. The presence of the latter gives clear *a priori* evidence that growth of the nerve-cord must proceed sub-terminally, and not terminally. Of the ventral longitudinal muscles the fifth has already been referred to; the sixth begins to form in the early pupa, and is therefore already well defined on the floor of the sixth segment of the first instar larva. Behind the sixth the seventh has already appeared, its formation taking place in the advanced pupa; this muscle, as Text-fig. 25 shows, intrudes well into the anal segment, and is a specially useful guide in following the development of the seventh segment during the first larval stadium.

Early in this stadium the epidermis of the anal segment and of the floor of the fifth and sixth segments undergoes intensive cell-proliferation, in consequence of which it grows still more in thickness, its cells becoming even more densely crowded, and imparting to the epithelium a markedly embryonic appearance; cf. fig. 131, Pl. X, with fig. 132, Pl. X (anal segment), and figs. 127 and 128, Pl. X (sixth abdominal segment). On the fifth and sixth segments the fourth and fifth pairs of legs now appear (fig. 35, Pl. III), and as these enlarge they soon become bent on themselves. The ganglion of the fifth segment meanwhile becomes separated from the epidermis, the vestiges of its 'ventral organs' being absorbed into it (Text-fig. 26 B). From the now greatly enlarged teloblastic ganglion a new ganglion has in turn been generated. It is the sixth abdominal. It is still in process of growth, for mitoses are encountered among its cells, and moreover, it is still connected with the sternal epidermis by the dwindling remnant of its 'ventral organ'.

While the fifth and sixth segments have thus been maturing, the seventh has begun to make its appearance (fig. 35, Pl. III). The intersegmental line delimiting it behind from the anal segment is already present, and on its tergal wall may be seen the initial thickening associated with the development of its trichobothrium. That the new segment has, like the sixth that preceded it, arisen out of cells that have proliferated from the anal segment, is proved by examining its ventral longitudinal muscle, for the muscle of the new segment (seventh ventral longitudinal) is already definable in newly emerged larvae, where its hinder end has attachment to the epidermis within the anal segment (Text-fig. 25). For the tergal wall of the segment the trichobothria give equally conclusive evidence, the new (third) trichobothrium taking origin within the limits of the anal segment (fig. 36, Pl. III; fig. 132, Pl. X).

In late first instar larvae terminal enlargement has proceeded to such an extent that there is much stretching of the chitin at the intersegments. The fourth and fifth legs have attained full segmentation, and the seventh segment has become much enlarged (fig. 37, Pl. III). The already existing (second) trichobothrium of the fifth segment has lost connexion with the cells that generated it, and a new second trichobothrium is in process of forming; and behind this, on the new seventh segment, the new (third) trichobothrium is growing out. Shortly before the first larval moult this trichobothrium acquires connexion with the teloblastic ganglion, into which the neuropilem has now extended. The ganglion of the sixth segment has meanwhile taken its place in the series of ganglia, having lost connexion with the sternal epidermis (Text-fig. 26 C).

Behind the ventral longitudinal muscle of the seventh segment another such muscle has appeared in late first instar larvae; as usual, it has its posterior attachment well within the anal segment, and thereby gives the first indication of the production of the eighth segment, which will, however, not be fulfilled until after the first moult.
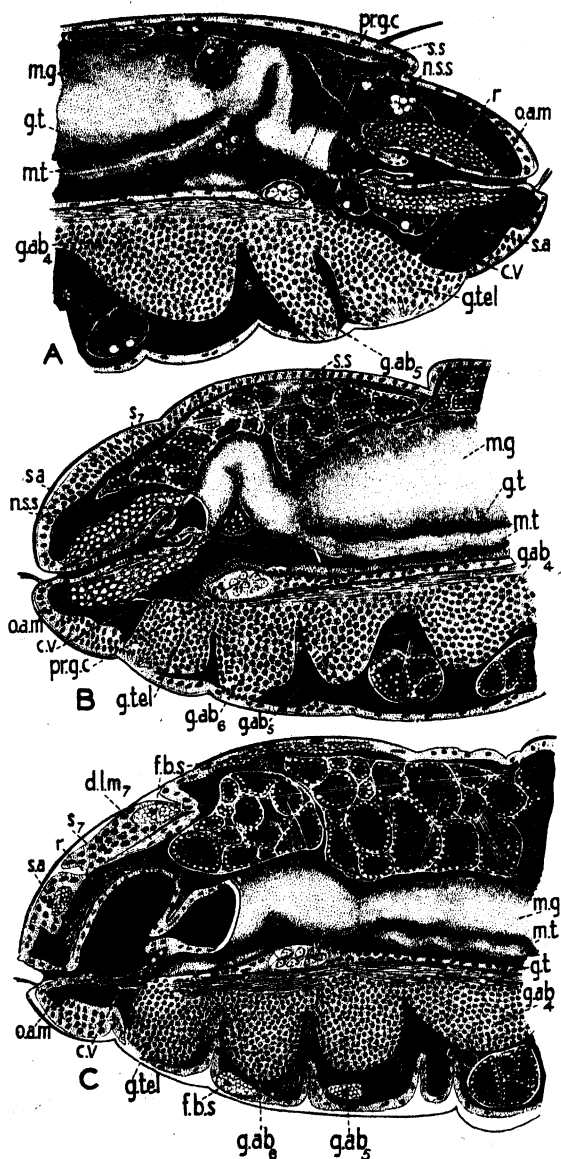
TEXT-FIG. 26. Internal views of hinder ends of the three first instar larvae shown in figs. 34, 35, 37, Pl. III.

A. Newly emerged larva. B, about half through first larval stadium. C, near end of first larval stadium. In A the new (fifth) ganglion has appeared; in B the sixth has formed, but is still in course of enlargement; in C the sixth has separated from the sternal epidermis. Fat-body is indicated as a transparent body; in A the large spherical inclu-

It is evident from the above description that two already formed segments have completed their development in the first larval stadium, while a single additional segment (seventh) has been added to the series. The ganglion of the latter is, in the early second instar larva, the teloblastic ganglion, which then again enlarges, thereby giving origin to the eighth ganglion. Rudiments of the new (sixth) legs also soon appear in the second larval stadium.

In the fourth instar larva the teloblastic ganglion still occupies the floor of the sub-terminal (now tenth) segment. No new ganglion is released from it, but it remains as the ganglion of the tenth segment. The new segment, which is produced after the moult (i.e. legless eleventh segment of the adult animal), is therefore devoid of a ganglion, its muscles, like those of the anal segment, being innervated from the preceding ganglion.

From the foregoing account it is evident that teloblastic segment formation in *Pauropus* proceeds in a much simpler manner than in Symphyla (Tiegs, 1945). Specially noteworthy is the evidence which it affords against the supposed bisegmental origin of the tergal shields. It has already been shown above (section 6, ii, A) that the second tergal shield of the first instar larva is the product of a single segment, and this now clearly holds for the new shields that develop in the larva. The fourth shield, for example, must be the product of the seventh segment alone, for the future eighth segment does not even exist in the second instar larva; and the fifth shield must clearly be the product of the ninth segment alone, for the future tenth segment has not yet appeared in the third instar.

3. *The Mesoderm*

In the advanced embryo the 'teloblastic mesoderm', whose formation has already been described in section 8 (viii), may be seen as two small clumps of cells that lie against the lateral body-wall behind the mesoderm of the fifth abdominal segment, i.e. within the limits of the anal segment. It is shown in fig. 87, Pl. 7. Out of this mesoderm is generated the musculature of the sixth and succeeding abdominal segments that develop in the larva; out of it develops also the median septum of neuroglia cells of the new nerve-ganglion; and finally it yields a small contribution of cells to the hinder end of the growing genital tube (see below, section 6).

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sions in the fat-body are residual yolk from the embryo; in B and C they have disappeared and smaller reserve granules are accumulating in great number. In C new (secondary) fat-body is arising from the epidermis. Note progressive development of the genital tube.

Lettering. c.v caudal visceral nerve; *d.l.m*$_7$ developing seventh dorsal longitudinal muscle; *f.b.s* secondary fat-body arising from epidermis; *g.ab*$_4$, $_5$, $_6$ fourth, fifth, and sixth abdominal ganglia; *g.t* rudiment of genital tube; *g.tel* teloblastic ganglion; *m.g* mid-gut; *m.t* Malpighian tube; *n.s.s* nerve from the second sensory seta (trichobothrium), passing to fifth abdominal ganglion; *o.a.m* occlusor ani muscle; *pr.g.c* primordial germ-cells; *r* rectum, cavity exposed; *s*$_7$ developing seventh segment; *s.a* anal segment; *s.s* second sensory seta (trichobothrium); the labelling line is directed to the clump of sense-cells at its base.

The development of the teloblastic mesoderm takes place along unexpectedly simple lines, without the production of somites. It begins in the early pupa; that this must be so is shown by the fact that the ventral longitudinal muscles and second oblique muscles of the future sixth and seventh segments are already present in the newly emerged larva (Text-fig. 25).

The early phases in the development of the teloblastic mesoderm consist merely of an enlargement of its mass, its proliferating cells becoming heaped up, in the young pupa, along the lateral wall of the anal segment, just behind the mesoderm of the fifth segment. From here the cells now spread backwards to form a complete lining to the floor of the anal segment, and also upward on to the roof of the segment, to form the Anlage of the new dorsal longitudinal muscle. These early phases will be understood by reference to fig. 129, Pl. X, and fig. 125 A and B, Pl. X; for orientation of the sections reference should be made to Text-fig. 9. Fig. 129 shows the position of the teloblastic mesoderm in relation to the mesoderm of the fifth segment; it represents one of the hindermost sections of a transversely cut young pupa, so that the hinder segments are necessarily cut 'horizontally' (cf. Text-fig. 9 for orientation). Figs. 125 A and B, on the other hand, are from a 'horizontally' cut series, the terminal segments being therefore transversely cut; the sections are immediately adjacent to one another, B being nearer the anus than A. In A note the lateral heaping up of the enlarging teloblastic mesoderm, and the development of some median mesoderm; in B note the complete lining of the floor of the anal segment by mesoderm.

The first muscle-Anlage to form out of the teloblastic mesoderm is the ventral longitudinal muscle of the developing sixth segment; it is present in 2-day pupae, and is first seen as a short column of cells that lies immediately behind the fifth similar muscle, and it intrudes into the anal segment. It may be seen in fig. 130, Pl. X, which should be compared with fig. 129, Pl. X; note that in the more advanced pupa shown in fig. 130 the fifth ganglion has become delimited from the teloblastic ganglion. In transverse sections the differentiating mesoderm may also soon be seen spreading down over the lateral wall of the enlarging sixth abdominal ganglion (still part of teloblastic ganglion); this is shown in fig. 126, Pl. X (the drawing should be compared with the right side of fig. 125 A, Pl. X, which is at exactly the same level, but from an early pupa). The vestige of teloblastic mesoderm that is not absorbed into the new segment remains as a conspicuous clump of cells on the floor of the anal segment (fig. 130, Pl. X), where it may also be seen in early first instar larvae (fig. 131, Pl. X). As described in the previous section, the epidermis of the anal segment again becomes, in the early first instar larva, a locus of intense cell-proliferation; the teloblastic mesoderm also again enlarges, and the above-described processes are repeated; cf. fig. 132, Pl. X, with fig. 131, Pl. X, and note in fig. 132 the Anlage of the new dorsal longitudinal muscle.

Only brief reference need be made to the further differentiation of the mesoderm allotted to the newly forming segments. That of the sixth segment

may serve as example. Figs. 125 A (right side), 126, 127, 128, Pl. X, represent this segment in successive stages of development, the sections being respectively from an early pupa, a late pupa, an early first instar larva, and a late first instar larva. In fig. 125 A, Pl. X, the mesoderm of the sixth segment is still part of the teloblastic mesoderm. In fig. 126, Pl. X, the Anlage of the ventral longitudinal muscle has appeared, and the mesoderm is spreading down the side of the ganglion to provide myoblasts for the sternal musculature, while other cells are beginning to spread a little way up the lateral body-wall to form the dorso-ventral muscles. In fig. 127, Pl. X, the ventral longitudinal muscle has completed its development (cf. Text-fig. 25), and a clump of myoblasts has become applied to the appendage Anlage; some of the dorso-ventral muscles are already in course of formation, and the dorsal longitudinal muscle is distinguishable. Finally in fig. 128, Pl. X, in which the fifth leg is forming, a great proliferation of the associated myoblasts has taken place, and these are being drawn into the cavity of the leg. In this figure the median mesoderm is no longer distinguishable, having become incorporated, in the usual way, into the new ganglion.

Specially noteworthy in the development of the teloblastic mesoderm is the complete absence of somites, in which respect *Pauropus* differs from *Hanseniella*, where even well-developed coelomic sacs are present in each developing teloblastic segment (Tiegs, 1945). As in *Hanseniella*, the larval mesoderm arises exclusively from the already formed mesoderm of the embryo; I have not been able to see any evidence that the new mesoderm arises directly from the epidermis of the growing zone, as described by Pflugfelder (1932) for the diplopod *Platyrrhacus*. Such formation of new mesoderm from the epidermis has been described for some annelids (*Lopadorhynchus*—Kleinenberg, 1886; *Spio*—Iwanoff, 1928); but in others (*Polygordius*—Woltereck, 1902; *Arenicola*—Lillie, 1905; *Aricia*—Iwanoff, 1928) it seems to arise exclusively from the embryonic mesoderm.

4. *The Alimentary Canal and Malpighian Tubes*

Both in the growing larva and in the adult animal an occasional dislodged mid-gut cell, with or without excretory content, may be seen within the mid-gut lumen. Beyond this there is no indication of any destruction of mid-gut epithelium, there being nothing comparable with the periodical renovation that is so striking a feature of the growing mid-gut of Symphyla and many insects.

Frequently, both in larvae and in adult animals, intestines are met that are entirely free from excretory substance; it would seem that, apart from its continuous slow ejection, this waste material may undergo a periodic but complete discharge from the mid-gut wall. In intestines which are thus freed of their stored excretion products, mitoses are occasionally seen among the epithelial cells, not only of the mid-gut itself, but also of the fore- and hind-gut (fig. 138, Pl. X). Actual cell counts in first instar and adult animals show a five- to sevenfold increase in the number of mid-gut cells. It is

probable that the cell-division which underlies this increase proceeds in sporadic outbursts, rather than continuously, for in those rare instances in which mitoses do occur they are present in relative abundance (e.g. a third instar larva with 180 mid-gut cells showed eleven in some phase of mitosis).

During the first larval stadium the Malpighian tubes enlarge considerably; their elongation does not, however, keep pace with that of the entire larva, so that, by the end of the stadium, their anterior ends, hitherto well within the second abdominal segment, have become withdrawn to the third. The distinction between the three regions, already indicated in the pupa (see above, section 9 (*b*) iv), is now very pronounced (fig. 134, Pl. X). These are: (i) a short proximal region of embryonic cells, enclosing a lumen that opens into the hind-gut; (ii) a longer median part of large pale and already markedly vacuolated cells; (iii) a shorter terminal portion of cells that present the appearance of gland-cells.

Throughout the larval period mitoses may be encountered in the growing zone at the base of each tube (fig. 135, Pl. X). The new cells, of course, become incorporated into the hinder end of the tube only, and there is no proliferation of cells within the terminal glandular part. The entire Malpighian tube of a late second instar larva is shown in fig. 135, Pl. X.

The Malpighian tube preserves this character until the adult condition is attained. Thereafter the already pale and weakly staining character of the main part of the tube becomes accentuated, and spreads into the terminal glandular portion. I have not been able to observe the time of closure of the bases of the tubes.

5. *The Fat-body*

In the embryo, as described above (section 12), the fat-body develops out of the yolk-cells. By the time the larva is ready to emerge this fat-body has become depleted of most of its reserves. In the newly emerged larva it is therefore found as a loose and irregular reticulum of cytoplasm, with scattered nuclei that display each an exceptionally large nucleolus, but without any sign of demarcation into separate cells. Unabsorbed yolk-grains, that have survived from the egg, are scattered rather sparsely throughout the fat-body (a few may be seen in Text-fig. 26 A).

As the larva begins to feed, reserve products start to accumulate again in the fat-body. These comprise the usual albuminoid grains and large spheres of fat. The fat-body thereby comes to acquire a much firmer texture than in the early larva (cf. figs. 127, 128, Pl. X). There is, however, never a sign of mitosis amongst its nuclei throughout the whole larval period, nor do any recognizable cell-partitions arise to break up the syncytial mass.

In newly emerged larvae about eighty nuclei can be counted in the fat-body; in the adult animal these have increased about fourfold. What is the source of this nuclear increase? In Symphyla there is ample evidence of mitosis in the fat-body of the growing larva (Tiegs, 1945); but in *Pauropus* I have not met it among several thousand nuclei examined. The possibility of

nuclear increase by amitosis cannot be excluded; there is, however, ample evidence of the formation of new fat-body from a quite unexpected source, namely the epidermis of the growing larva.

In newly emerged larvae the epidermis is, on the whole, rather thin, and is composed of cells with comparatively small nuclei (fig. 127, Pl. X). I can find no evidence of associated mesodermal cells, the formation of new fat-body from which might lead to the false impression of its derivation directly from the epidermis. During the first larval stadium some of the epidermal cells begin to enlarge, acquire bigger nuclei and clear cytoplasm, and are now in the first phase of differentiation into fat-cells (fig. 128, Pl. X). They are found mainly along the tergal body-wall, though a few are also present below the nerve-cord. A rather more advanced phase in the development of the new fat-body is shown in fig. 136, Pl. X. The enlarging epidermal cells have now acquired a highly alveolar cytoplasm, while the adjacent small epidermal cells are beginning to close in over them. The developing fat-cells thereby become pressed out of the epidermis; it is, however, noteworthy that at this phase of their development they are still part of the epidermis itself, for they lie external to the basement membrane.

There follows upon this the condition shown in figs. 137 and 138, Pl. X. The newly forming fat-body, easily distinguishable by its delicate lace-like reticulum and absence of reserve material, has grown in bulk, and has begun to assume a syncytial character, while renovation of the epidermis above it is almost complete.

Finally, the new fat-body separates from the epidermis; large fat-vacuoles appear within it and the albuminoid reserves become more abundant. The already formed fat-body thereby becomes displaced to the sides of the enlarging abdominal cavity, the new fat-body occupying the space between the alimentary canal and the dorsal body-wall, while there is also a smaller amount under the nerve-cord. In larvae in which the old fat-body contains ample reserves, the contrast between it and the new fat-body is very marked (fig. 145, Pl. XI). But as reserve material begins to accumulate in the new fat-body, the distinction between the two is gradually lost.

The above-described formation of new fat-body from epidermis was quite unexpected. In insects and in Symphyla growth of the fat-body in the larva may be attended by mitosis in already formed fat-body, or new fat-cells may arise from dormant mesodermal cells. As far as I know the only record of new fat-body formation out of epidermis is that of Rössig (1904) for certain gall-forming wasps (Cynipidae).

6. *The Reproductive Organs*

(a) *Anatomy of Adult Organs.* The structure of the reproductive organs has already been described by Schmidt (1895), Kenyon (1895), and Silvestri (1902); there is, however, considerable lack of agreement, particularly for the male organs. The accounts of Schmidt and Kenyon refer to *Pauropus huxleyi*, that of Silvestri to *Allopauropus brevisetus*.

(i) *Female* (Text-fig. 27). The ovary is a very thin-walled unpaired sac, lying between the nerve-cord and the intestine; it extends throughout the fifth to eighth segments, and, when replete with eggs, even into the tenth. The statement by Schmidt that the eggs probably develop from the epithelial wall of the ovary is incorrect, for a localized germarium is present. The latter is unpaired, and is situated on the floor of the ovarian tube near its middle. (This is not general for Pauropoda; in an undescribed species which I have the germarium occupies the hindermost end of the ovary.) It is more conspicuous in animals with eight leg-pairs than in adults, and in the latter tends to disappear with advancing age (see Text-fig. 27 A). The eggs do not lie loose within the cavity of the ovary, but are contained within a very thin follicular membrane, by which they are attached to the ovarian wall. From four to six eggs may simultaneously undergo enlargement by accumulation of yolk, the expanding ovary pressing upon the adjacent organs. Between the enlarging eggs there may be abundant smaller eggs in which yolk has not yet begun to accumulate.

At its anterior end the ovary passes into a remarkable thick-walled cylindrical glandular duct, through which the eggs must pass on their way to the oviduct. I shall speak of it as the 'ductus glandularis'. It extends from the fifth to the third abdominal segment. It is composed of tall columnar gland-cells, and is invested externally by a strongly developed layer of circular muscle-fibres. When empty its cavity is, except for a narrow median channel, hardly visible; closer inspection reveals, however, an almost completely collapsed triradiate lumen between its thick epithelial walls (Text-fig. 27 C). Whether its function is to secrete the egg-shell, as Schmidt has suggested, is uncertain; in none of the females, with large eggs in the ovary, that I have examined are the eggs provided with a shell.

There is only a single oviduct and it may lie either to right or to left of the nerve-cord, around which it bends on its way to the genital atrium. It is much thinner than the ductus glandularis, and provides a surprisingly narrow channel for the passage of the large eggs. Its walls, evidently highly distensible, are lined by cubical epithelium, but are not glandular in character (Text-fig. 27 B).

The oviduct opens into the side of the genital atrium. This is merely a slight depression in the floor of the third segment, just behind the second legs, there being no genital sclerites associated with it. I have sections from one animal in which the genital atrium, distended with some glandular material, presumably from the ductus glandularis, is invaginated deeply into the floor of the segment.

Into the genital atrium, immediately above the orifice of the oviduct, opens a single receptaculum seminis, a short, thick-walled, flask-shaped organ, lined by cubical epithelium, and often filled with sperms (Text-fig. 27 B).

(ii) *Male* (Text-fig. 28). There are four large rounded testes lying, one behind the other, above the alimentary canal below the fourth to sixth tergal
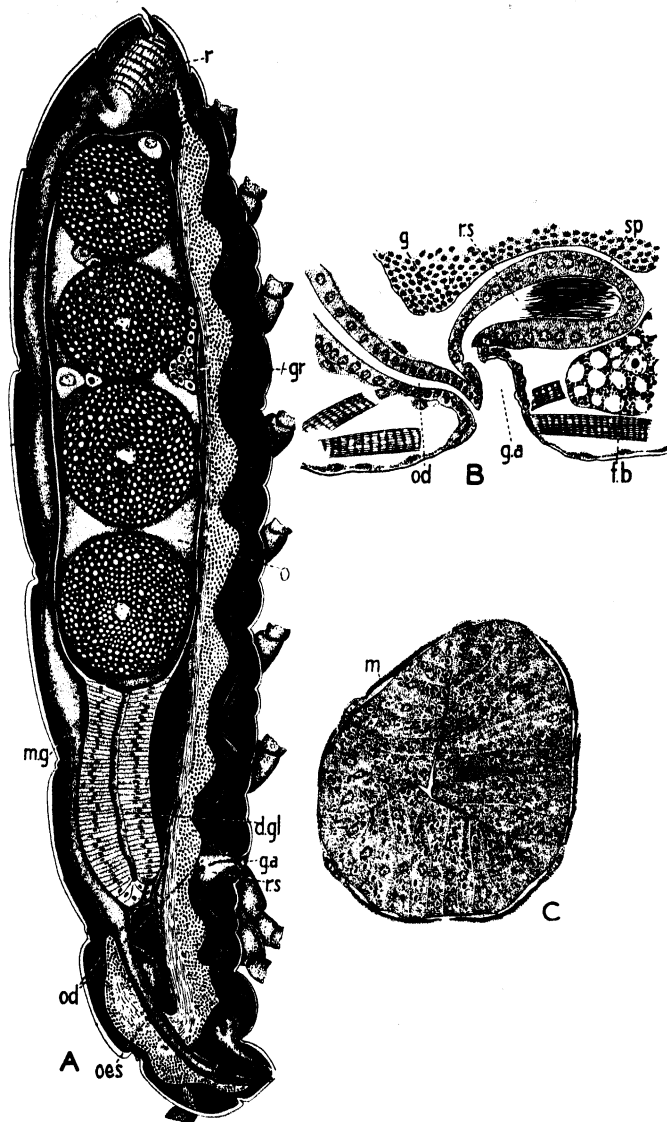
TEXT-FIG. 27. Structure of adult female reproductive organs; from an animal in which the germarium has almost disappeared.

A. Internal view of adult female. B. Transverse section through floor of genital segment, showing genital atrium, and the openings into it of the receptaculum seminis and oviduct. C. Approximately transverse section through ductus glandularis.

Lettering. d.gl ductus glandularis; *f.b* fat-body; *g* ganglion; *g.a* genital atrium; *gr* vestige of germarium; *m* muscle sheath; *m.g* mid-gut; *o* ovarian tube; *od* oviduct; *oes* oesophagus; *r* rectum; *r.s* receptaculum seminis; *sp* sperms in receptaculum.

shields. Each testis is a hollow sac, completely enclosed by germinal epithelium, and the interior is filled with masses of developing sperms. Investing the germinal epithelium is a thin, almost imperceptible, membrane, which is the wall of the genital tube itself. The four vasa deferentia that arise from the four testes pass down, two on either side, below the intestine, where they continue forward as four vesiculae seminales.

The vasa deferentia are easily distinguishable from the vesiculae by the orientation of their sperm content, whereas in the vesiculae the sperms are disposed quite at random (Text-fig. 28 C, B). The walls of the vasa deferentia are composed of very large flattened cells, while externally there is a thin investing sheath. The vesiculae, whose walls are markedly glandular, are often distended with sperms. Within the sixth segment the four vesiculae become confluent at their tips, and from the common chamber the two exit channels arise. Developmentally the latter are found to form from the epidermis alone, and though differentiated into three distinct regions, may therefore conveniently be spoken of as the ejaculatory ducts. Following Kenyon (1895) we may refer to the three regions as the posterior, glandular, and anterior ejaculatory ducts respectively. The posterior ducts are quite short and narrow. The glandular section of the ducts are not unlike the unpaired 'ductus glandularis' of the female; they are, however, rather thinner, the nuclei lie at the bases of the cells, and an investing muscle sheath is absent. The narrow lumen of each lodges a long structureless filament of unknown meaning (Text-fig. 28 B). It is probable that the function of the glandular ducts is to secrete a viscous matrix in which the sperms are transferred to the female, for sperms within the receptaculum seminis of the female are always enveloped in a thick secretion which is not visible in the vesiculae. The anterior ejaculatory ducts pass to right and left of the nerve-cord to the penes, which lie just median to the second coxae (Text-fig. 24 B).

(b) *Development*. In the pupa the reproductive organs are still in a very rudimentary condition (cf. above, section 11). The future genital tube consists of a narrow string of cells, resting in a median groove along the roof of the nerve-cord, and extending from the fifth abdominal segment a short distance into the third (Text-fig. 9). Not more than about a dozen cells are present, and of these most are aggregated around the single primordial germ-cell (or exceptionally two germ-cells) located within its hinder end (fig. 139, Pl. XI). Behind the germ-cell the cells of the genital tube merge with the median mesoderm of the developing new segments. Sexual distinctions are not yet evident.

In this condition the reproductive organs are found even in newly emerged larvae. During the course of the first larval stadium the primordial germ-cells and cells of the genital tube begin to divide. In consequence the future genital tube grows in thickness. In late first instar larvae from four to six primordial germ-cells are present within it, forming a bulge at its hinder end, but of a lumen there is yet no sign (Text-fig. 26).
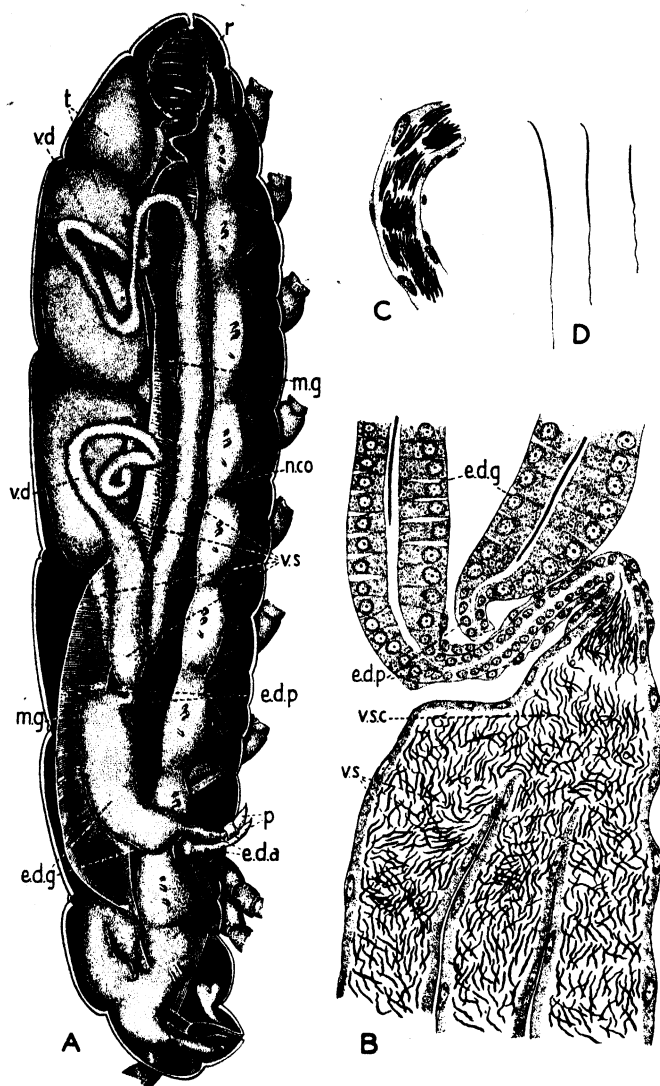
TEXT-FIG. 28. Structure of male reproductive organs.

A. Internal view of adult male to show general plan of reproductive system. B. Fragment from a 'horizontally' sectioned adult male, showing vesiculae seminales and portion of ejaculatory ducts; only three of the four vesiculae are present in the section. C. Fragment of vas deferens. D. Mature sperms.

Lettering. e.d.a, e.d.g, e.d.p anterior, glandular, and posterior portions respectively of the ejaculatory ducts; *m.g* mid-gut; *n.co* nerve-cord; *p* penis; *r* rectum; *t* testis; *v.d* vas deferens; *v.s* vesicula seminalis; *v.s.c* common chamber formed by fusion of vesiculae seminales.

Connexion with the exterior is established by the ingrowth of a pair of ducts from the epidermis a little postero-medial to the bases of the second legs. The development of the ducts often takes place in first instar larvae, though in many cases it is delayed till well into the second larval stadium. They first become apparent as a pair of small epidermal thickenings out of which arise a pair of ingrowing cords of cells (fig. 140, Pl. XI), which, passing upward and a little backward, bend medially round the ventral longitudinal muscle, and fuse with the anterior tip of the genital tube over the roof of the nerve-cord. The genital tube has, by this time, grown markedly in thickness, but even now does not display a lumen.

Even well into the second, and often third, larval stadia, no sexual distinction is evident; it is particularly noteworthy that the epidermal exit ducts do not give the expected clue, for in these earlier larvae they are paired in both sexes, though, in the adult female, only one of the two ducts survives. The structure of the gonad prior to the onset of sexual differentiation is shown in fig. 138, Pl. X, the drawing being from a sagittal section of a second instar larva. The genital tube, still devoid of a lumen, is now a compact cord of cells. The germ-cells, much increased in number, form a bulge at its hinder end, and have now moved back into the sixth abdominal segment. It is evident from the position of these cells that the genital tube must have become elongated behind; and this terminal elongation seems to be still in progress, for posterior to the germ-cells the genital tube passes directly into the undifferentiated median mesoderm of the developing new (seventh and eighth) abdominal segments. As far as I have been able to observe, terminal growth of the genital tube ceases after the second larval moult.

Sexual differentiation becomes apparent first in the gonad itself; and since the testis departs more than the ovary from the primitive condition, it is the males that are at first distinguishable with certainty.

(i) *Male.* In some larvae differentiation of the gonad-rudiment into the four testes is already in progress before the end of the second larval stadium, though generally it is deferred till well into the third. The earliest phase in the differentiation of the testes that I have obtained is shown in Text-fig. 29 A; a section taken through its hinder portion is drawn in fig. 145, Pl. XI. It is taken from a late second instar larva, and shows the developing testes arising as two pairs of lateral thickenings that are growing up to the side of the mid-gut from the anterior and the posterior ends respectively of the gonad rudiment, which itself now lies mainly in the sixth segment. In transverse section the wall of the gonad displays an epithelial character, with much mitosis amongst its cells. Within the four developing testes germ-cells are plainly distinguishable by their large nuclei; the intervening region, with relatively small nuclei, evidently represents the wall of the genital tube itself, and out of it the vasa deferentia will eventually form; but as fig. 145, Pl. XI, shows clearly, its epithelium merges into the developing germinal epithelium of the testis Anlage itself.

A later phase in the differentiation of the male gonad, taken from a third instar larva, is shown in Text-fig. 29 B; a section through the anterior, middle, and hinder regions respectively is depicted in fig. 146, A, B, and C, Pl. XI. The anterior pair of testes have grown high up around the mid-gut, and extend almost to the dorsal body-wall (fig. 146 A). The hinder pair of testes are also markedly enlarged, but their dorsal displacement is not very advanced, for they still lie to the side of the intestine (fig. 146 C). Between the anterior and posterior pair of testes the genital tube is reduced to a comparatively narrow band of cells, with just perceptible lumen (fig. 146 B). Within the testes the germ-cells have greatly increased in number, and among these a few (indicated by x in fig. 146, Pl. XI) are already in meiotic prophase.

In fourth instar larvae development of the testes proceeds apace. The anterior pair has become much enlarged (Text-fig. 29 C), sections through them (fig. 147, Pl. XI) showing that the enlargement is due to distension of their cavities. The testicular cavity is lined by germinal epithelium, external to which is a thin membrane, often recognizable only by its flattened nuclei, and presumably the product of the original sheath of mesoderm (genital tube) which invested the primordial germ-cells. Within the germinal epithelium mitoses are abundant, and many spermatocytes, with nuclei in meiotic prophase, have been released into the cavity of the testis. The posterior pair of testes have also much enlarged, and within them also spermatogenesis is proceeding; they still lie, however, to the side of the intestine.

The vasa deferentia have now also appeared, and the vesiculae seminales are in course of formation. The vasa deferentia arise from that part of the genital tube which originally included the germ-cells. As the latter become concentrated within the four enlarging testes, the intervening part of the genital tube, now freed from germ-cells, becomes reduced in thickness. The displacement of the anterior pair of testes into a position above the intestine has the effect of drawing out the adjoining part of the genital tube into the anterior pair of vasa deferentia, which, however, remain connected with the genital tube below the intestine; this will readily be seen in fig. 146 A, Pl. XI. That portion of the genital tube which still joins the anterior pair of testes with the posterior pair has now become reduced to a narrow strip with just-perceptible lumen (fig. 146 B, Pl. XI; Text-fig. 29 B). In fourth instar larvae this median band is seen to have divided longitudinally into the two vasa deferentia of the hinder pair of testes, and these soon become widely separated from one another (Text-fig. 29 C; fig. 147, Pl. XI).

Anterior to the region of the gonad, i.e. in the fourth and fifth abdominal segments, the genital tube has grown markedly in width. As the four vasa deferentia become better defined, the whole of this widened part of the genital tube proceeds to divide into four separate tubes that lie side by side above the nerve-cord. The splitting spreads from behind forwards; fig. 148, Pl. XI, is drawn from the actual region of division. In these four separate tubes we have the rudiments of the vesiculae seminales; the outer pair is
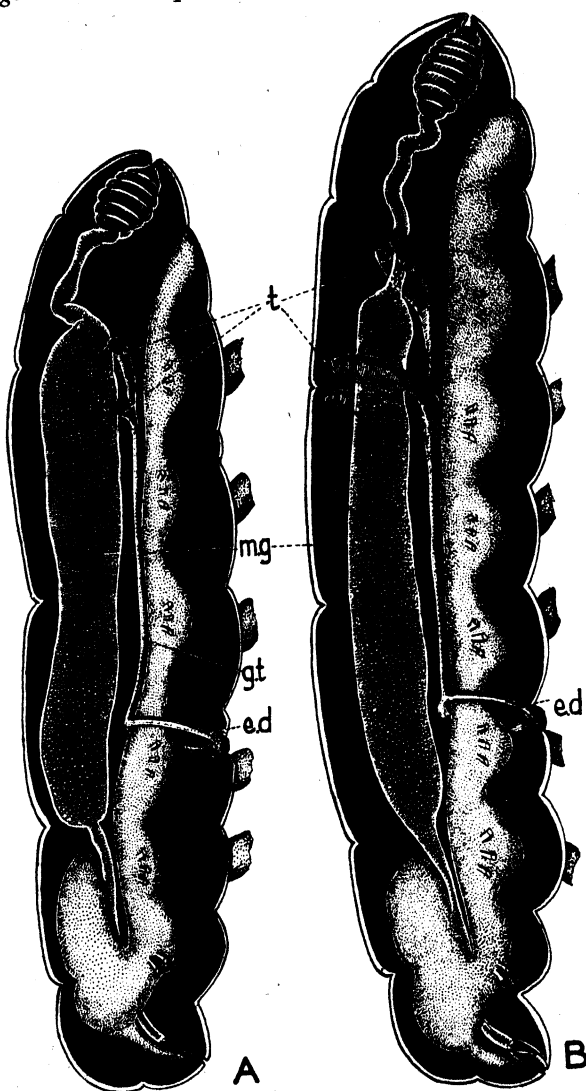
TEXT-FIG. 29. Differentiation of male reproductive organs.

A. Initial phase (from a late second instar larva) showing the four rudimentary testes develop-
ing out of the genital rudiment. The epidermal exit ducts have already become associated
with the anterior end of the genital tube. Sections through such a larva are shown in
figs. 141, 145, Pl. XI. B. Later phase (from a third instar larva). For section see figs. 145
A, B, C, Pl. XI. C. From a fourth instar larva, showing later phase in differentiation of the
vasa deferentia, and the beginning of splitting of the genital tube into the four vesiculae
seminales. For section see fig. 147, Pl. XI. D. From a fourth instar larva, showing still
later phase of development. The anterior pair of testes is completely dorsal in position,
the posterior pair in process of becoming so, but alinement of the testes has hardly begun.
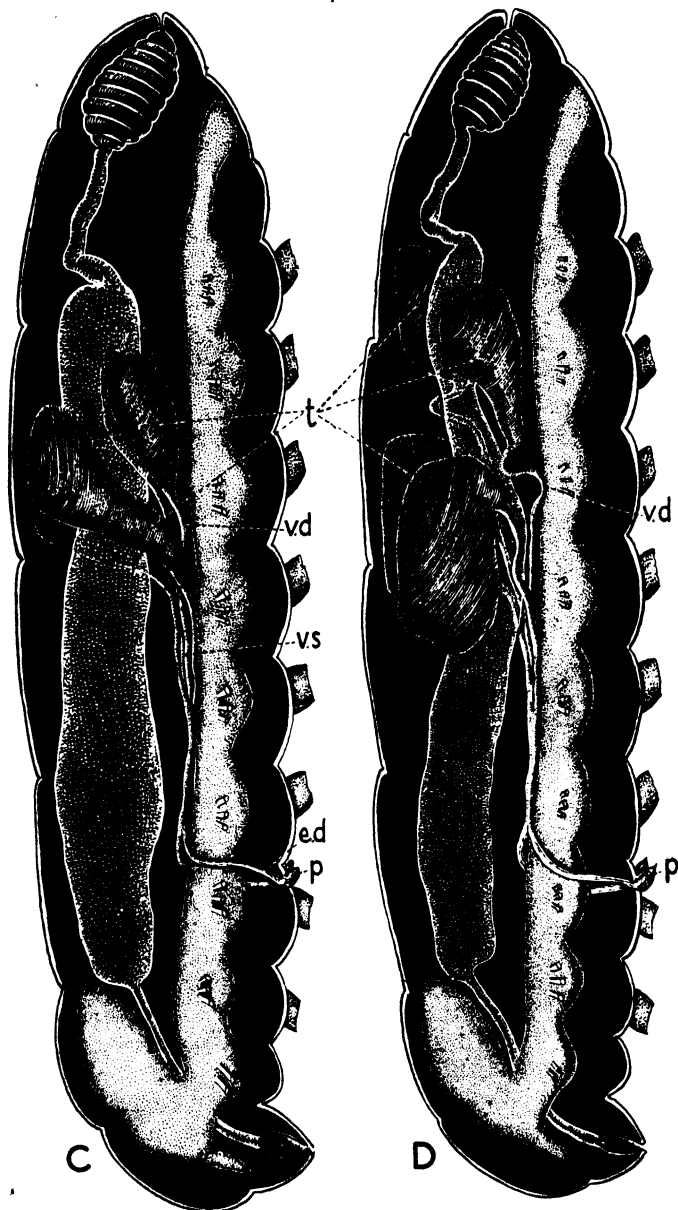
Differentiation of the epidermal exit ducts into the ejaculatory ducts has also not yet started.
                            All four figures drawn to scale.

Lettering.   *e.d* undifferentiated exit ducts (rudimentary ejaculatory ducts); *g.t* genital tube; *m.g* mid-gut; *p* penis; *t* testes; *v.d* vasa deferentia; *v.s* vesiculae seminales.

connected, through the anterior pair of vasa deferentia, with the anterior pair of testes, the median pair with the hinder pair of testes.

A still later phase in the development of the testes is shown in Text-fig. 29 D. The testes have enlarged further, and the anterior pair has begun to assume its definitive form, with the vasa deferentia arising from their postero-lateral walls. Twisting of the vasa deferentia is now also beginning. In this larva the final alinement of the four testes, one behind the other, has hardly begun; nevertheless even before the final larval moult this alinement is attained.

Late in the fourth larval stadium spermatogenesis becomes accelerated, and all phases from early spermatocytes to apparently mature spermatozoa are visible within the cavities of the enlarging testes. A testis from such a larva, cut in sagittal section, is shown in fig. 150, Pl. XI. The vesiculae seminales have now also begun to increase in thickness, their cells enlarging and acquiring an obviously glandular texture of cytoplasm (fig. 149, Pl. XI); but in none of the fourth instar larvae that I have examined do they ever show any sperm-content.

The ejaculatory ducts alone are of epidermal origin, for they develop out of the paired ingrowing cords of cells whose formation in an earlier larval instar has already been described above. Throughout the second and third larval stadia they undergo considerable thickening, there being much evidence of mitosis among their cells; but a lumen is not distinguishable except perhaps near their epidermal attachments (fig. 141, Pl. XI). Not till late in the fourth larval instar does structural differentiation set in. By separation of cells each now develops a lumen throughout its length. The inferior portions of the ducts remain rather thin-walled, and so form the 'anterior ejaculatory ducts'. The middle part becomes markedly thickened, and out of this forms the glandular portion of the ejaculatory ducts (fig. 142, Pl. XI). I have not been able to make direct observations on the development of the short thin posterior ejaculatory ducts, but as they participate in the terminal bifurcation of the genital ducts, it is probable that they too must be assigned to the epidermal portion.

The two penes are already present in fourth instar larvae (Text-fig. 29 C and D; fig. 142, Pl. XI). This points to the possibility of a precocious sexual maturity in some males, as in Symphyla; but although apparently fully mature sperms are present in the testes of some fourth instar larvae, I have never seen any that have been released into the vesiculae seminales. The penes develop, in third instar larvae, by thickening of the epidermis around the bases of the ejaculatory ducts. Just prior to the next moult they begin to protrude from the surrounding epidermis, and so become prominent in fourth instar larvae.

(ii) *The Female.* In its development this departs much less from the condition of the gonad-Anlage than is the case for the male, and consequently females are not distinguishable as early as the latter; in no instance have I been able to recognize the female before the third larval stadium. In such larvae the gonad-Anlage has somewhat thickened, but otherwise retains its

earlier features except that, among the nuclei of the germ-cells, we can occasionally recognize prophase stages of meiosis (fig. 151, Pl. XI). Later, during the third larval stadium, these meiotic prophases become much more abundant, while at the same time a number of cells with small nuclei appear among the germ-cells (fig. 152, Pl. XI). It is from these cells that the future follicles, within which the eggs are held in the ovary, are developed. It is most unlikely that they can have arisen from the germ-cells; it is much more probable that they grow into the ovary from the ovarian wall, for they are connected with the latter, and have nuclei of similar appearance.

The ovary in such larvae lies within the sixth and seventh abdominal segments. As in the male the genital tube has now lost all connexion with the median mesoderm of the developing hinder end of the larva, so that it does not acquire any additional cells from this source. In front of the ovary the genital tube has markedly thickened, but does not, at this period, show any noteworthy difference from that of the male. The entire ovary, together with proximal part of the genital tube, from a larva cut in 'horizontal' section, is shown in fig. 153, Pl. XI.

During the following larval stadium enlargement of certain oocytes begins. Therewith the ovary itself grows still more in size, its most anterior tip lying well within the fifth segment, while its hinder end may intrude a little into the eighth. The enlarging oocytes lie in two rows along the lateral walls of the ovary, and may even bend up a little to envelop the lateral walls of the intestine, giving to the ovary the false appearance of a paired organ. Between the two rows of enlarging oocytes, on the floor of the ovary, and within the sixth and seventh segments, lies the mass of unenlarged germ-cells, some in mitosis, others in various prophases of meiosis; we may now speak of this as the germarium (fig. 154, Pl. XI).

In the adult animal the ovary enlarges still further, as the number of enlarging oocytes increases, and may extend almost into the fourth segment. The germarium, on the other hand, decreases in size as its cells are gradually released into the cavity of the ovary, and eventually disappears. Text-fig. 27 shows an adult female with dwindling germarium.

The ductus glandularis develops from that portion of the genital tube that lies in the fourth and fifth segments, anterior to the ovary, and is wholly mesodermal. Its rudiment, from a fourth instar larva, is shown in fig. 155, Pl. XI, its point of junction with the epidermal exit duct being very clearly seen. Already in this undeveloped condition it displays an outer thin investment of flattened cells, from which presumably the muscle-coat later develops. Sometimes the transformation of this rudiment into the fully formed duct is complete even before the final moult; on the other hand, I have often seen 'adult' animals, with nine leg-pairs, in which the duct is still imperfectly developed. The expectation that the ductus glandularis might have a similar origin to the glandular ejaculatory duct of the male, which it closely resembles, has not been fulfilled; the ductus glandularis is rather the equivalent of the vesiculae seminales.

Of the two epidermal exit ducts which are present in earlier larval instars, only one survives, and this may be either the left or the right duct. Degeneration and final disappearance of the opposite exit duct takes place in fourth instar larvae (fig. 143, Pl. XI). There is no evidence that the receptaculum seminis is a vestige of the degenerated exit-duct, for fourth instar larvae are sometimes encountered in which the second duct has completely disappeared, before there is even a sign of the receptaculum. The latter arises as a thickening of the base of the surviving exit duct (oviduct)—fig. 143, Pl. XI, but later acquires an opening into the genital atrium, close beside that of the oviduct, but quite distinct from it (fig. 144, Pl. XI). The atrium is merely a depression of the epidermis between the bases of the second coxae.

(c) *Gametogenesis.* This will be treated only very briefly, since a detailed consideration of the nuclear phenomena is not within the scope of this work.

(i) *Spermatogenesis.* Throughout the later larval instars, and particularly in the fourth, mitoses appear in great abundance in the germinal epithelium, and this has the effect of further enlarging the testes and of replacing cells that have been liberated into their cavities. In adult animals the frequency of mitosis declines.

When we examine the contents of the testes, we see all phases in the development of sperms, from early meiotic prophases to the completed gamete. These are not scattered singly through the testes, but lie in clumps, and within these clumps the progress of spermatogenesis is markedly synchronized (fig. 150, Pl. XI). This appears to be due to the fact that patches of germinal epithelium, and not individual cells, are released into the testicular cavities. Especially in fourth instar larvae is this release of masses of spermatocytes, with nuclei in early meiotic prophase, met with; it is evidently in progress in the upper part of the testis shown in fig. 150, Pl. XI.

After release from the testes, the mature sperms are stored in the vesiculae, which may be distended with them. The heads of the sperms (Text-fig. 28 D) are rod-shaped bodies, often with a slight bend at the tip, and range in length from 13μ to 24μ. (The above measurements are of the same order as those given by Schmidt (1895) for *Pauropus huxleyi*. In *Allopauropus brevisetus*, according to Silvestri (1902), the sperms are many times this size. In an undetermined Australian species, to which I have referred several times above, similar enormous sperms are present.) The tail is about the length of the head, but can be seen only in sharply stained preparations. Living sperms examined in saline often display typical swimming movements, but even in such active forms the tail is quite invisible.

(ii) *Oogenesis.* In the third instar larva a considerable proportion of the germ-cells in the ovary are in some recognizable prophase of meiosis. In many others the nuclei are in a resting condition. Occasionally a true mitosis is seen. Between the germ-cells are the future follicular cells, easily distinguishable by their small size (fig. 152, Pl. XI).

In the following larval stadium, as already described, the more laterally situated germ-cells begin to enlarge, each being now enclosed within its

own sheath of follicular cells (fig. 154, Pl. XI). Their nuclei are in the germinal vesicle condition. The median row of smaller germ-cells may be regarded as germarium. But after the last larval moult an increasing proportion of its cells, also, are found in the germinal vesicle condition, even though the phase of enlargement has not yet set in. Early phases of meiosis are now scarce, and true mitoses very rare (fig. 1, Pl. I).

In certain of the enlarged oocytes yolk then begins to accumulate, smaller oocytes, still without yolk, lying singly or in small groups between the enlarging cells (fig. 2, Pl. I; Text-fig. 27). Prior to the laying down of yolk, a deeply staining body, presumably a 'yolk-nucleus', may be met with in the oocytes (fig. 2, Pl. I).

After the discharge of the egg from the ovary, other laterally placed germ-cells within the 'germarium' begin to enlarge, to repeat the process. Eventually the whole germarium disappears.

The nuclear phenomena which follow upon the germinal vesicle phase have been described above (see Part 1, Emb. Dev., section 1).

I have not been able to make any observations on the development of the chorion. The fact that none of the ovarian eggs which I have seen showed even a rudiment of a chorion supports the suggestion made by Schmidt (1895) that the chorion may be secreted on to the eggs as they pass along the ductus glandularis.

Affinities of the Pauropoda

Although taxonomic work on these minute and fragile creatures has brought to light peculiar genera like *Eurypauropus*, *Brachypauropus*, and *Decapauropus*, the group still remains a singularly homogeneous one, whose members reveal no clear indication of close affinity with any of the other forms of myriapods. Lubbock (1868), who looked upon paucity of appendages as a primitive and ancestral feature, regarded the Pauropoda as 'a link not only connecting the chilopods and diplopods together, but also bridging over to a certain extent the great chasm that separates these forms from other articulates'; and when, in 1879, Ryder discovered the remarkable *Eurypauropus*, he, too, emphasized the annectant character of the group, for it seemed to him to link the diplopods, chilopods, hexapods, and even, owing to the branching antenna, the crustaceans.

Adopting the position of the gonopore as the basis of classification, Kennel (1891) was the first to suggest a dichotomous line of descent of the tracheate arthropods, in which either an anteriorly situated, or a posteriorly situated pair of segmental organs of some primitive *Peripatus*-like ancestor had become utilized as the exit ducts of the reproductive organs; the one line of descent gave origin to the Pauropoda, Symphyla, Diplopoda, and Arachnida, the other to the Chilopoda and Insecta. Pocock (1893) proposed a similar scheme, but wisely excluded the Arachnida, and his classification of the 'tracheate arthropods' into Progoneata and Opisthogoneata is now generally accepted. The affinities of the Pauropoda within the Progoneata have,

however, remained uncertain. Schmidt (1895) correctly recognized the evidence of structural simplification in their minute bodies, and regarded the Pauropoda and Symphyla as simplified offshoots of the 'protodiplopod' stem. (I have shown in a previous paper (Tiegs, 1940) that the evidence for vestigial diplopody in Symphyla is invalid.) Kenyon (1895) united the Pauropoda and Pselaphognatha as sub-orders in the order Protodiplopoda. In Lankester's (1904) classification the Symphyla and Pauropoda are included in the Diplopoda.

If current notions on the heterogeneous character of the 'Myriapoda' are well founded, comparison of *Pauropus* with any but the progoneate forms must be futile. Thus in Lankester's classification (1904) the Onychophora, Diplopoda (including Pauropoda and Symphyla), and Arachnida are conceived as 'terrestrial offshoots of successive lower grades of primitive aquatic Arthropoda which are extinct', while 'the Hexapoda, and with them, at no distant point, the Chilopoda, have branched off from the Crustacean main stem as specialized terrestrial lines of descent'. Even the more conservative schemes of Kennel and Pocock can leave no room for comparison with opisthogoneate groups. There is, however, little doubt that the remarkable anatomical characters of the Symphyla invalidate these classifications; for our present knowledge seems to justify Ryder's (1800) original contention that the Symphyla are an annectant group, which weld together the Insecta, Diplopoda, and Pauropoda into a group of relatively closely related Arthropoda. Into the evidence for their insectan affinities it is not necessary here to enter, as this has recently been reviewed by Calman (1936), Imms (1936), and Tiegs (1940); but I would, as newer evidence, point to the remarkable embryonic 'dorsal organ' common to Symphyla, Collembola, and *Campodea* (Tiegs, 1942a, 1942b), and not known, so far, in any other arthropod. Their diplopod affinities are perhaps less striking, for their embryology does not support Schmidt's (1895) contention, accepted by several authorities, that their abdominal segments present a vestigial diplopody; but the manner of segmentation of the egg and of mesoderm formation, and the presence of such organs as the exsertile vesicles and spinning glands (both present in the diplopod order Nematophora), segmented mandibles, organs of Tömösvary, fat-body, proctodaeal Malpighian tubes, as well as peculiarities in structure of the brain and gonads, are features which point to more than a merely general relationship with Diplopoda.

In view of these facts it is surprising that a single character of uncertain morphological value should, as a criterion of classification, be held to outweigh so much evidence of affinity between the progoneate and opisthogoneate groups. Does the position of the gonopore really have the great significance that is generally assigned to it? It is, of course, true, that within the various natural groups of myriapods its position is constant. The notion at the bottom of the classification is, however, the retention of a forwardly situated pair of ancestral coelomoducts to serve as gonoducts in the Progoneata, and of a posteriorly situated pair in the Opisthogoneata. For the latter the scheme seems to be well justified; for in *Scolopendra* (Heymons,

1901) and in various Orthoptera and Dermaptera (Wheeler, 1893; Heymons, 1895; Wiesmann, 1926; Roonwal, 1937) the gonoducts do indeed develop, in the embryo, out of mesodermal coelomoducts. This seems to hold also for *Peripatus* (Sedgwick, 1887), though Kennel (1886) asserts that here the ducts are chiefly ectodermal. In the Progoneata, on the other hand, in which evidence from the Symphyla and Pauropoda is now available, it is plain that the exit ducts from the genital tubes are not coelomoducts at all, as had been assumed, but are ectodermal ingrowths. For the Diplopoda evidence on this point is not yet available.

The gonopore of Progoneata seems, therefore, in contrast to that of Opisthogoneata, to have the status of a secondary opening, and it is not unlikely that it was acquired as an adaptation to their anamorphosis; for if the genital segment has become involved in teloblastic growth, the need has arisen for the formation of a new gonopore, remote from the zone of growth, unless its formation may be deferred till after the completion of anamorphosis. As long ago as 1895 Kenyon, recognizing the anomalous position of *Scolopendrella* (Symphyla), wrote: 'The animal truly shows many chilopod and some thysanuran features, but until it can be shown from a study of its ontogeny that the genital ducts are already fairly well formed before the posterior segments, it is safe to conclude that the position of the genital opening on the fourth body-segment, together with other characters, indicates an affinity nearer the Diplopoda than to any other group of Arthropods.' Actually, not only are the genital ducts of Symphyla 'already fairly well formed before the more posterior segments', but they may even be functioning (Tiegs, 1944). In *Pauropus*, also, they arise long before the hinder end of the animal has completed its development, but there is no evidence in this case that the gonads are functioning before the completion of anamorphosis. For the Diplopoda no observations on the development of the genital ducts seem to have been made; the remarkable observations of Verhoeff (1926) on 'intercalated males' (Schaltmännchen) of Julidae (with indefinite segment number) have shown, however, that terminal accretion of segments does indeed continue after sexual maturity, though this does not hold for species with definite segment number. The existence of anamorphic chilopods, with terminal genital opening, does not invalidate this line of argument, for at least five instars intervene between completion of anamorphosis and the attainment of sexual maturity, and it is in these instars that the genital segment and external genitalia develop (hemianamorphosis of Verhoeff, 1902–25). For the Protura, in which three new segments are added behind during anamorphosis, no relevant observations seem to have been made.

It would appear, then, that at present the classification of 'tracheate arthropods' into Progoneata and Opisthogoneata, implying as it does two independent lines of descent from an ancestor with multiple gonoducts, is not sufficiently well founded to invalidate a comparison of *Pauropus* with other than progoneate forms. In the following discussion a more general comparison will therefore be drawn.

(a) *General Features of Development.* The segmentation of the egg recalls that of Symphyla and most Diplopoda and Collembola. In the phases which ensue—a blastoderm, within which a germ-band becomes defined, from which, in turn, scattered cells separate away to form the mesoderm—we see the distinctive features of myriapod-insect ontogeny. There can, indeed, be no question of the ingrowth of mesoderm at a blastopore, as in *Peripatus* or Crustacea. Yet in *Pauropus* a modified gastrula can be distinguished preceding the blastoderm, but with characters which give a possible clue to the path of evolution of the more specialized ontogeny without a gastrula that prevails in other myriapods and insects. This question has already been discussed (see Emb. Dev., section 5).

If there is any close affinity between myriapods and higher Crustacea, as some authors contend, these early developmental phases might be expected to reflect it. That the segmentation of the egg may at times be very similar, as in *Scolopendra* and *Astacus*, must be conceded; such resemblances are, however, largely governed by the yolk-content of the egg. On the other hand, the peculiar manner in which the ectoderm and mesoderm develop in higher Crustacea, from ectodermal and mesodermal teloblasts, is never encountered in any myriapod or insect embryo. Moreover, in Crustacean embryos there seems to be an almost total suppression of coelomic sacs, only a few well-attested cases of diminutive coelomic sacs having been recorded (*Estheria*, Cannon, 1924; *Hemimysis*, Manton, 1928). It is true that in *Pauropus* the coelomic sacs are also not well developed; but this is almost certainly a simplification, associated with the absence of a cardiac Anlage (see Emb. Dev., section 8, ix). In the embryos of all other myriapods and primitive insects so far examined, we find very complete series of coelomic sacs, which in size rival even those of *Peripatus*.

The presence of a gastrula in *Pauropus*, even though it be a much modified gastrula, must be adjudged a primitive feature. The failure to develop completely specialized vitellophages is also primitive, for, as in the yolked species of *Peripatus* (Sheldon, 1887–8; Evans, 1901) and in Symphyla, all its 'yolk-cells' are subsequently used in the formation of adult tissues. These tissues in *Pauropus* are the mid-gut epithelium and fat-body. The development of the fat-body out of 'yolk-cells' is particularly significant; it arises in a similar way in Symphyla and probably Diplopoda, but in chilopods and insects is derived from the mesodermal somites.

The absence of a ventral flexure in the germ-band is noteworthy. In all other myriapods and in insects a ventral flexure appears, being sometimes precocious (Symphyla, Diplopoda) or delayed (Chilopoda, Insecta).

The absence of embryonic membranes is not unexpected, for they are not found in any other myriapod, and are a product of evolution within the class Insecta.

The 'dorsal organ' of the embryo is not the highly specialized organ of Collembola, Symphyla, and *Campodea*, but is a smaller and simpler structure, recalling rather the 'dorsal organ' of Crustacea (see Emb. Dev., section 14, vii).

(b) *Post-embryonic Development.* The most striking feature of the post-embryonic development of Pauropoda is the anamorphosis, which they have in common with Diplopoda, Symphyla, Protura, and some Chilopoda. What significance attaches to it?

It is probable that precocious emergence from the egg, with deficient segmentation, is merely an adaptation to the deficiency of yolk available to the growing embryo. Some authors contend that the ensuing anamorphosis is a repetition of ancestral history, in which a line of few-segmented ancestors grew in length by the sub-terminal acquisition of new segments; this is, indeed, the central idea in Tillyard's (1930) theory of the evolution of myriapods and insects. The laws of metamerism are, however, too insufficiently known to permit any such deduction; what significance can attach to the condition of the larva at eclosion, when within the single family Scutigerellidae, some species emerge with six pairs of legs, others with seven? There is, in fact, no sure evidence as to whether the ancestor of myriapods was a few-segmented (Tillyard), or, as Lankester (1904) urges, a multi-segmented arthropod; probability favours the latter view, since paucity of segments, both in extinct and surviving arthropods, is quite exceptional. The deficient abdominal segmentation of Collembola is almost certainly a case of arrested development (Imms, 1936); that of Pauropoda is probably the same (see Post-emb. Dev., section 1).

Since two orders of chilopods, and all insects except Protura, develop without anamorphosis, is the latter to be adjudged a secondary acquisition of those myriapods that display it, or is the reverse the case? The occurrence of anamorphosis in all the primitive surviving myriapods points to the likelihood of its being an archaic feature, and not a secondary acquisition. (According to recent studies by Fahlander (1938) the multi-segmented chilopods without anamorphosis are the least primitive members of the group.) But this evidence can hardly be regarded as conclusive; in particular it throws no light on the question whether the anamorphosis was complete, or a 'hemianamorphosis' of the chilopod type. It is important in this connexion to observe that emergence from the egg in a partially developed condition is not confined to the anamorphic myriapods; its prevalence throughout the metabolic insects is the basis of Berlese's theory of larval forms (see Imms, 1937). It is reasonable to expect that a larva, forced to precocious emergence through lack of nutriment in the egg, should find it advantageous to have well-developed anterior parts, on which the sense and feeding organs are borne, and relegate the completion of its hinder end to the post-embryonic phase; but it is disconcerting to find that the metabolic insects, confronted with the same problem, have adopted the alternative of emerging with a complete outfit of imperfectly developed segments. Possibly the difference has been imposed by the character of the ontogeny: in the embryo of *Hanseniella agilis* the germ-band divides into a head and five abdominal segments, to which four additional segments are successively added by sub-terminal growth before eclosion, and judging by Silvestri's (1903) figures, this type of

development characterizes also the diplopods (*Pachyjulus*). This can also be recognized in *Pauropus*, though here the teloblastic growth in the embryo is limited to one segment; and it is clearly also the case in *Peripatus*, as the figures of Sedgwick and Kennel show. In *Scolopendra* (Heymons, 1901) and insects, on the other hand, segmentation of the germ-band proceeds along different lines; the latter attains its full length, after which segmentation spreads along it, but there is no teloblastic growth of segments in the egg. The extreme of precocious emergence in the first type of ontogeny is provided by *Pauropus* and some diplopods; for the second type of ontogeny it is found in the remarkable 'protopod' larvae of some parasitic Hymenoptera, in which even external segmentation of the abdomen is suppressed.

(c) *The Head.* The structure of the head-capsule and its appendages is of special importance for the assessment of affinities.

In the head of *Pauropus* specialized features are easily recognized, such as the peculiar branched antennae with their globuli and flagella, the deeply embedded unsegmented mandibles adapted to a semi-fluid diet, and the suctorial mouth-parts and oesophagus. But beneath these specializations there is evidence of a very primitive construction of the head; for while in Chilopoda, Symphyla, and Insecta the head-capsule is composed of a procephalon to which have been added a mandibular, maxillary, and second maxillary segment, in the Pauropoda the latter segment has not been cephalized, but remains distinct from the head as the collum segment.

In *Peripatus* the 'head' consists of three segments, each furnished with a pair of large coelomic sacs and segmental organs in the embryo, and with a pair of ganglia (Kennel, 1886; Sedgwick, 1887; Evans, 1901); the acron, equivalent of the annelid prostomium, is not separately demarcated, but the 'frontal processes' may perhaps represent its antennae (Korschelt and Heider, 1899). Following Heymons (1901) most morphologists are agreed that the procephalon of myriapods and insects is formed by the welding together of an acron and three segments (pre-antennary, antennary, and pre-mandibular), and it would therefore appear to be the equivalent of the 'head' of *Peripatus*. Snodgrass (1938) has recently proposed a radical change in our conception of head-segmentation in myriapods and insects, the pre-mandibular segment being regarded as the first true segment, and the whole region anterior to this as acron. The absence of any trace of intersegmental grooves in the entire procephalon is held to outweigh the doubtful testimony for segmentation which the coelomic sacs and ganglia offer. It must be acknowledged that in insect embryos the delineation of the segments, and particularly of the acron, is largely conjectural; on the other hand, Heymons (1901, pp. 34, 37) states quite explicitly that in *Scolopendra* intersegmental grooves are present delimiting not only the gnathal segments, but also the antennary and pre-antennary segments and acron. To regard the coelomic sacs of the procephalon as 'ontogenetic structures' for the accumulation of waste products is probably to under-estimate their real significance; in a generalized myriapod like *Hanseniella* both pre-antennary and antennary coelomic sacs perform

a normal role in developmental processes, in that both play an essential part in the development of the dorsal blood-vessel, and even in insects the antennary coelomic sac plays this role, though the pre-antennary is usually reduced to a vestige. The hope that so primitive a myriapod as *Pauropus* might throw some light on this question has not been fulfilled, for intersegmental grooves appear too late, and all the somites are vestigial. Ferris (1942), on the other hand, believes that the component segments of the heads of insects can be identified by sutures even in the adult head-capsule; but since intersegmental lines are not present in the procephalon of the embryo, it is surprising, though perhaps not impossible, that they should be present in the adult. It is noteworthy that these adult sutures transect a region of the head from which the protocerebral ganglion develops. In *Scolopendra*, where the evidence seems to be clear, this ganglion develops from the acron (Heymons, 1901), in which case the sutures cannot possibly be true intersegmental lines. A re-examination of the development of a chilopod head is very desirable, as it seems to offer the best chance for deciding these questions.

Evolution in the Myriapoda has been attended by the successive merging of three gnathal segments with the procephalon. In *Pauropus* we seem to have a very specialized descendant of a primitive stock of myriapods with only two gnathal segments, the 'lower lip' protruding from between the maxillae. We may speak of these as DIGNATHA. (The name has already been used by Lankester (1904) though with a different implication. It is noteworthy that Imms (1936) assigned only two gnathal segments to the hypothetical 'Protomyriapoda'.) In the Chilopoda, Symphyla, and Insecta cephalization has proceded a segment further, and they may be spoken of as TRIGNATHA. In the embryology of Symphyla we have evidence of the relatively recent incorporation of the third gnathal segment (second maxillary) into the head, for that segment appears first as part of the abdomen, being separated from the head by a deep intersegmental groove; and even in insects the abdominal character of the mesoderm of this segment has long been recognized (Wiesmann, 1926). In the Chilopoda the maxillae, especially the second maxillae, still retain, to a surprising degree, the character of ambulatory appendages, and it is the appendages of the first abdominal segment (poison jaws) which have undergone the greatest specialization. The lower lip of the pre-oral cavity in Chilopoda is formed by the first maxillae, fused, as Heymons (1901) has shown, with a diminutive maxillary sternite, the fused bases of the second maxillae (sterno-costal plate) lying well behind the pre-oral cavity. In Symphyla and Insecta, on the other hand, the first maxillae operate within an enlarged pre-oral cavity, a new 'lower lip' being formed by the fusion of the second maxillae (labium). This is the highest grade of elaboration undergone by the gnathocephalon in the Myriapoda–Insecta; following Snodgrass (1938), we may speak of its members as LABIATA.

The position of the Diplopoda is still uncertain. The lower lip is here formed by the gnathochilarium. It has at times been claimed (e.g. Carpenter, 1905) that two pairs of maxillae can be distinguished in the adult organ. But

embryology alone can provide the clue to its composition; yet there is a complete lack of unanimity amongst embryologists as to its manner of formation. The contradictory accounts of its development have already been set out (Emb. Dev., section 6, v); it will suffice here to repeat that while the most recent work, that of Pflugfelder, contends that it develops by a fusion of first and second maxillae, most writers (Metchnikoff, Heymons, Silvestri, Lignau) state that only the first maxillae enter into its formation. Heymons does not even refer to an incorporation of the post-maxillary segment into the head, but Silvestri and Lignau agree that the sternum of that segment becomes the 'hypostome' of the gnathochilarium. Snodgrass (1938) includes the Diplopoda in the Labiata, and in my earlier paper on the development of Symphyla (Tiegs, 1940) I expressed a similar view. Experience of the development of *Pauropus*, the germ-band of which is almost identical with that figured by Silvestri (1933) for the diplopod *Archispirostreptus*, leads me to suspect that this view is wrong, and that the Diplopoda are in reality Dignatha; for the possible incorporation of a post-maxillary sternite, without appendages, into the head, is not comparable with the cephalization of a whole segment as in Chilopoda, much less still with the formation of a true labium. (In the only Palaeozoic diplopod whose mouth appendages have been described (the Permian *Acantherpestes*) there is apparently no gnathochilarium, but a pair of mandibles and a single pair of maxillae (Fritsch, 1901).) But if Pflugfelder's observations should eventually prove correct, then the case for the inclusion of Diplopoda among the Labiata will have been proved. A really critical investigation of the development of Diplopoda is much needed.

The primitive organization of the head of *Pauropus* is expressed also in its musculature. Snodgrass (1928), in discussing the problem of the insect tentorium, has shown how the sternal adductor muscles must, in the first instance, have arisen from the hypopharyngeal apophyses, but that in Chilopoda the muscles of the mandibles have secondarily taken origin from a ligamentous bridge between the apophyses, while 'in Diplopoda, Crustacea, and Apterygota groups of the adductor fibres from the mandibles have lost their sternal connexions, and have united with each other by a median ligament to form a dumb-bell muscle between the two jaws'. In Symphyla I have found (1940) that the primitive condition postulated by Snodgrass has survived in the mandibular musculature; and this proves to be the case also in *Pauropus* (see Emb. Dev., section 15). On the other hand, in the great development of the oesophageal dilator muscles that take origin from the apophyses, we have one of those remarkable specializations that are so frequent in *Pauropus*.

In the primitively constructed head of *Pauropus* we seem to have the clue to some hitherto-unsolved problems in the morphology of the mouth-parts of other myriapods and insects. The maxillae, as we have seen (Emb. Dev., section 6, ii, B), are more generalized appendages than was formerly thought; for a lacinia, stipes, and cardo are distinguishable, even though a palp and galea are lacking. The cardo in this case is not provided with muscles, and

may therefore be looked upon, not as a part of the appendage itself, but as a pleural sclerite; and it is not unlikely that this homology may be extended to cardines in general. Snodgrass (1928), it may be recalled, has already pointed to the difficulty of seeing in the cardo a distinct basal segment of the appendage. It is probable, also, that in the maxillae with their associated intermaxillary plate, we have the forerunner of the complex gnathochilarium of diplopods; for the lateral parts of the gnathochilarium show an unmistakable resemblance to maxillae, in which cardo and stipes are distinguishable, while the central part may well be derived from the intermaxillary plate, the hypostome alone being of post-maxillary origin. In the gnathochilarium of *Polyxenus* these components are especially evident. In this primitive diplopod, according to Carpenter (1905), superlinguae (maxillulae) are also present, but these seem to be unknown in other diplopods.

It would also appear that with the formation of a new 'lower lip' in the Labiata, the primitive 'lower lip' of Dignatha, as exemplified in *Pauropus*, has survived as the hypopharyngeal apparatus; superlinguae are common to both, being derived in both cases from the mandibular epidermis, and the median lobe (hypopharynx proper, lingua) is formed in Labiata mainly from the maxillary sternite, though there is evidence that the mandibular and even labial sternite may contribute to its formation.

(*d*) *The Abdomen*. The chief problem in the abdomen relates to the nature of the segments: are they simple segments or diplosegments? Kenyon long ago observed that while in *Eurypauropus* 'the diplopod condition is indubitable', on the other hand, in *Pauropus* alternate legs seem 'to come between the dorsal plates, thus much less clearly bearing evidence of a diplopodial segmentation'. Attems (1926) nevertheless writes 'there is recognisable a developmental tendency for two segments to be united into double segments by a common tergite', and this view may fairly be said to express current opinion. Verhoeff (1934) makes the extraordinary assertion that the segments of *Pauropus* are quadruplosegments, produced by a fusion of two diplosegments, which have each been reduced beyond the stage of reduction attained by Symphyla; but I have shown in a previous paper (Tiegs, 1940) that the embryology of Symphyla does not support the view that the abdominal segments of these animals are reduced diplosegments, and there is certainly nothing in the embryology of *Pauropus* from which Verhoeff's statement can gain any support.

In *Brachypauropus* each leg-bearing segment, except the first, is said to have one tergal scute, and a diplopod condition is therefore excluded in this genus. But in the remaining genera of Pauropoda there is only one such tergal shield for every two leg-bearing segments. If animals are examined that have died in a contracted condition, the impression is undoubtedly given that the tergal shields are diplotergites that cover two separate segments; but if the animals have died in an extended condition, then the tergal shields seem to be the products of alternate segments only (Text-fig. 24 A). Embryology, which alone can decide the question, confirms this latter interpretation

(see above, Emb. Dev., section 6, ii, A; Post-emb. Dev., section 2). The commonly accepted opinion that we have in the abdomen of *Pauropus* an incipient diplopody proves therefore to be an error; the condition of the segments may be compared rather with that of some chilopods (*Lithobius* and especially *Scutigera*) in which there is a tendency for reduction in the tergal region of alternate segments. In the oldest-known myriapod, the Silurian and Devonian *Archidesmus* (Peach, 1882, 1889), a similar reduction in the tergites of alternate segments is found. In the Diplopoda, on the other hand, the segments are welded together in couples, which have a common tergite. This remarkable condition may have been brought about either by the duplication of parts within single segments, or by the coupling of segments; the presence of two separate sternites in the diplosegments of the extinct and primitive Palaeozoic Macrosterna (Fritsch, 1901) is sugges- tive of an incomplete fusion of simple segments.

In one important respect, however, the Pauropoda seem to show affinity with the Diplopoda. In both groups the post-maxillary segment appears to be a legless collum segment. In Pauropoda the evidence is very clear; here the collum segment is a complete ring, and even in the embryo is devoid of any rudiments of appendages (the so-called vestigial legs of the collum segment of *Pauropus* are not developed from appendage rudiments in the embryo—see Emb. Dev., sections 6, ii, A; 14, v). In Diplopoda, also, the post-maxillary segment seems to be devoid of any rudiments of appendages in the embryo (Heymons, Silvestri), Pflugfelder's statement to the contrary standing in need of confirmation; but the collum segment, which develops out of the post-maxillary segment of the embryo, is not in diplopods a com- plete ring, for its sternite seems to become incorporated into the head as the hypostome. In this respect the collum segment of Diplopoda shows an advance in specialization over that of *Pauropus*. Should the legless post- maxillary segment prove to be a general feature of all Diplopoda, it will provide most weighty evidence of affinity of these animals with Pauropoda; for in no other myriapod is this peculiar condition found (the so-called collum segment of some Symphyla is a post-labial segment). In the primitive diplopod *Polyxenus*, according to Reinecke (1910), a collum segment is not present; but the interpretation of adult segments in diplopods is notoriously uncertain, and needs the support of embryological evidence.

The legs, on the other hand, except for the smaller trochanter and the jointed tarsus, resemble those of Symphyla rather than of Diplopoda; for the pre-femur of Diplopoda is not present, nor do we find the close approxima- tion of opposite coxae that is so characteristic of these myriapods (Text-fig. 24 B). But with Diplopoda and Symphyla the legs of Pauropoda share one primitive feature: opposite legs move in unison, whereas in chilopods and insects their action is alternating.

Exsertile vesicles (coxal sacs) have not hitherto been described for Pauro- poda. The small papillae on the collum segment, commonly regarded as reduced legs, are, however, probably organs of this type (cf. Emb. Dev.,

section 14, v); and, like the exsertile vesicles of Symphyla, they develop from the remains of the 'ventral organs'. In Diplopoda true exsertile vesicles are met with; from Scudder's (1882) drawing it is evident that they were well formed in the huge Carboniferous *Euphoberia*, and amongst surviving groups they are prevalent in the orders Nematophora and Cholobognatha. They have not been found in any chilopod, but occur throughout the Symphyla, and, amongst primitive insects, in Diplura as well as in *Machilis*. The supposed abdominal appendages of Protura have, at their ends, a form of exsertile vesicle, and such a vesicle is also present on the ventral tube of Collembola. Whether the vesicles of Protura and Collembola, situated, as they are, at the ends of what seem to be appendages, are comparable with exsertile vesicles that arise from the abdominal wall, is uncertain; but apart from these doubtful cases, the widespread occurrence of exsertile vesicles in other myriapods and primitive insects points to the essential unity of these groups.

The function of these organs is unknown. They have been variously regarded as adhesive, water-absorbing, or even excretory; current opinion favours the view that they are respiratory, though there is no real experimental evidence for this. It is stated that in *Machilis* they are extruded in a warm damp atmosphere (Haase, 1889), but whether to absorb moisture or oxygen is uncertain. Nutman (1941) has shown that in Collembola the vesicle at the end of the ventral tube has a water-absorbing function. The exsertile vesicles of Symphyla seem to have a similar function, as the following observations on *Hanseniella agilis* show. A batch of sixteen animals were placed overnight in a petri dish on soil that had been moistened with water containing a little dissolved 'light green' dye. The following morning the animals were anaesthetized and examined with a binocular microscope. In eight animals the entire series of vesicles was coloured bright green; in one animal only a few were coloured. The coloration was quite selective for the exsertile vesicles, except for an occasional discoloration of the chitin of the distal leg-joint. It is therefore evident that the animals had been applying the opened vesicles to the moistened soil. Many of the animals had also succeeded in taking moisture into the alimentary canal. In four, both alimentary canal and exsertile vesicles were coloured; in five, only the latter; three had used only the alimentary canal, while in the remaining four there was no evidence of any water-intake at all. In *Campodea fragilis* I have never obtained any discoloration of the vesicles, though the alimentary canal in all cases soon became green. In *Pauropus*, also, the 'exsertile vesicle' of the collum segment was never found discoloured; in this animal there is evidence for direct absorption of water through the general chitin, for the interior of the distal leg-segments is commonly discoloured. Judging by the work of Oudemans (1888), the exsertile vesicles of *Machilis*, also, are not used for the absorption of water.

(*e*) *The Glands*. For purposes of phylogenetic discussion, interest centres chiefly in those salivary glands that are of mesodermal origin. These are

generally regarded as derivatives of segmental organs, after the manner of the great salivary glands of *Peripatus*. Of these glands there are two in *Pauropus*, the pre-mandibular and the maxillary glands.

The presence of a functional pre-mandibular gland in the remote ancestors of insects and chilopods had been suspected by Wheeler (1893) and Heymons (1901), and its actual occurrence in *Pauropus* does not, therefore, come as a surprise. Its presence on the third cephalic segment suggests, indeed, a direct affinity with the salivary gland of *Peripatus*. In *Pauropus* the adult gland shows no vestige of its original tubular character, and the absence of an end-sac is particularly noteworthy. The survival of this gland in *Pauropus* is probably correlated with the inturning of the lateral margin of the clypeus (cf. section 6, ii, B), by which means the orifice of the gland has become enclosed within the enlarged pre-oral cavity. In the Symphyla pre-mandibular glands, complete with end-sacs, are present up to the time that the larva leaves the egg, after which they degenerate, the end-sacs alone surviving as the great tubular nephrocytic organs (Tiegs, 1940). It will be recalled, in this connexion, that the end-sacs of the nephridia of *Peripatus* have a nephrocytic function (Bruntz, 1904a). In chilopods the 'lymphoid tissue' of the pre-mandibular segment, and, in insect embryos, the sub-oesophageal bodies, nephrocytic in appearance, and also derived from the pre-mandibular mesoderm, seem to be the vestiges of this organ; it was indeed these tissues which first suggested to Wheeler and Heymons the former existence of a pre-mandibular segmental organ. In the weevil *Calandra* the sub-oesophageal body survives even in the adult insect, and here its nephrocytic action in response to injected ammonia carmine is readily displayed (see Emb. Dev., section 10, ii). In Collembola the 'head-kidney' described by Hoffman (1908) seems to be a related body.

The presence, in *Pauropus*, of a mesodermal maxillary gland, furnished with a nephrocytic end-sac, is also not unexpected, for such a gland is present in Symphyla. In the diplopod *Julus*, also, the tubular maxillary gland is of mesodermal origin (Heathcote, 1888); in *Glomeris* the nephrocytic character of the end-sac of the maxillary gland has been shown by Bruntz. Among chilopods, *Scolopendra* seems to be lacking in mesodermal glands (Heymons), but in *Scutigera* and *Lithobius* there is present a maxillary gland whose structure recalls a segmental organ (Fahlander, 1938), but its development still needs elucidation. Most of the other cephalic glands of chilopods are epidermal ingrowths (Heymons, 1901), in which respect they resemble the cephalic glands of pterygote insects.

Amongst Collembola and Thysanura we find the remarkable tubular glands whose development from mesoderm has in one case, *Isotoma*, been proved (Philiptschenko, 1912). These glands have end-sacs, which display a nephrocytic action towards injected ammonia carmine (Bruntz, 1904b; Philiptschenko, 1908). But it is noteworthy that they are not maxillary but labial glands, and as such are not present in *Pauropus*. According to Fahlander (1938) labial glands occur also in certain chilopods and in the symphylid

Scutigerella immaculata; but in *Hanseniella agilis*, which I have examined carefully, they are not present.

As the labial glands are mesodermal and are furnished with typical end-sacs, they are regarded as surviving archaic segmental organs. That they should be present in the labial rather than maxillary segment is surprising, for the labial segment is looked upon, with good reason, as a recent addition to the head, and can therefore hardly be expected to have retained its segmental organ. Have we here, perhaps, an instance of Lankester's 'seventh law' of metamerism (translation of heterosis)?

(*f*) *The Alimentary Canal.* In the simplicity of its structure, the alimentary canal of *Pauropus* resembles that of other myriapods, of primitive insects and of *Peripatus*, and differs most markedly from the specialized gut of most Crustacea, with its cephalic 'stomach' and associated 'liver' and (in Malacostraca) complex fore-gut.

The presence of Malpighian tubes, associated with the hind-gut, is also a myriapod feature; similarly named tubes from a group of Crustacea (Amphipoda) are hardly comparable with them, for they are mid-gut derivatives.

The Malpighian tubes present us with a peculiar problem. In *Peripatus* they are not present, the most effective organ of excretion being the mid-gut epithelium (Manton, 1937). They are found in most myriapods and insects; and even in myriapods there seems no reason to doubt their excretory function (Plateau, 1876). Yet we find a recurring tendency for these organs to degenerate again and even disappear, the mid-gut epithelium or even fat-body assuming the role of excretory organ. In *Pauropus*, for example, it is clear that the mid-gut epithelium is the principal excretory organ, as in *Peripatus*; in *Pauropus huxleyi* Malpighian tubes are said to be absent (Schmidt, 1895); in *P. silvaticus* they are markedly degenerate and do not open into the hind-gut; in *Allopauropus brevisetus*, according to Silvestri (1902), they are present but small, and only in *Eurypauropus* do well-formed tubes occur (Kenyon, 1895). Again, in Symphyla a pair of well-formed tubes is present, and these show at least some evidence of excretory function, for they eliminate indigo-carmine when this is injected into the blood (Tiegs, 1944); yet urate concretions are quite absent in their lumen, but accumulate in great quantity in the fat-body. In the chilopod *Lithobius* the mid-gut epithelium seems to eliminate waste products, even though Malpighian tubes are present (Manton, 1937). In Collembola, where Malpighian tubes are absent, special 'urate cells' are present in the fat-body, and the mid-gut epithelium also seems to exercise an excretory function (Folsom and Welles, 1906). In *Campodea*, where sixteen very diminutive Malpighian tubes are said to be present, urates accumulate in great quantity in the fat-body, as is the case also in *Japyx*, where Malpighian tubes are apparently absent. The meaning of these facts is not clear.

(*g*) *Blood and Respiratory System; Fat-body.* The absence of a heart and of blood-vessels in *Pauropus* must clearly be attributed to degeneration, and

is presumably correlated with dwarfing of the body. The little movement that the blood is capable of is probably imparted to it chiefly by peristalsis of the intestine.

The absence of tracheae is probably also a simplification, cutaneous respiration sufficing in so small a body. Collembola, which rival *Pauropus* in minuteness, also rely upon cutaneous respiration, only one family (Sminthuridae) having tracheae. In the minute Protura the tracheal system is also, when present at all, greatly reduced.

The presence of fat-body is a feature which *Pauropus* shares with all other myriapods and with insects; it is not found in Crustacea. In all insects so far examined, and in chilopods, it develops from cells which separate from the mesodermal somites. Its development in *Pauropus* out of 'yolk-cells' in the embryo is, however, not unexpected, for it has a similar origin in Symphyla (Tiegs, 1940), while Heathcote's rather meagre description for *Julus* points to a similar origin. But the development of secondary larval fat-body out of epidermis (Post-emb. Dev., section 5) seems to be without parallel in myriapods.

The phylogenetic origin of fat-body in myriapods is unknown, there being no comparable tissue in *Peripatus*. Its development out of yolk-cells in primitive myriapods suggests that it may have arisen by the persistence of yolk-bearing vitellophages beyond the egg and pupoid phases into the larva.

(*h*) *Reproductive Organs.* Here interest centres chiefly in the evaluation of the progoneate condition as a criterion of classification. The manner of development of the gonoducts does not support the view that they are, as in *Peripatus*, chilopods, and insects, the remains of segmental organs; and since they arise long before the hinder end of the abdomen has completed its development, it is not unlikely that their position remote from the zone of growth, is a secondary adaptation to anamorphosis. This question has already been discussed.

It remains only to refer briefly to the gonads themselves. The unpaired condition of the gonad-Anlage is surprising, as is also the failure of the coelomic sacs to participate directly in its development. It is impossible to assess the meaning of this beyond suspecting that it is bound up with the general decline of the coelomic sacs in *Pauropus.*

The structure of the ovary, with its parietal[1] germarium and scattered oocytes, each encased in a follicular epithelium, recalls that of other myriapods (*Lithobius*, Tönniges, 1902; *Polydesmus*, Effenberger, 1909; *Hanseniella*, Tiegs, 1945) and of Collembola (Willem, 1900; Imms, 1906), but is wholly unlike that of all true insects.

The location of the testes dorsal to the alimentary canal is quite different from that of diplopods and Symphyla, and resembles rather that of *Peripatus* and chilopods; the resemblance is, however, misleading, for both in *Peripatus* and chilopods the testes develop from the dorso-lateral lobes of the coelomic

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[1] This is not general for Pauropoda; reference has already been made above (Post-emb. Dev., section 6) to a species of *Pauropus* with terminal germarium in the ovary.

sacs, while in *Pauropus* they migrate into this position from below the alimentary canal.

(*i*) *The Nervous System and Sense Organs.* Beyond the presence of only two component ganglia in the sub-oesophageal ganglion (dependent upon the occurrence of only two gnathal segments in the head), the fully formed nerve-cord of *Pauropus* does not display any features worthy of note.

In the embryo, however, the developing ganglia are associated with 'ventral organs'. These peculiar structures were first described and named by Kennel (1886) from the embryo of *Peripatus*, where they were later also observed by Sedgwick (1888). In *Peripatus* the 'ventral organs' are bulging thickenings of the ectoderm below the developing ganglia; they present, in their middle, a gentle depression, from which the nuclei recede, but toward which they are orientated. These features also distinguish the 'ventral organs' of *Pauropus* and Symphyla (Tiegs, 1940). In Symphyla they are not themselves an important source of ganglion-cell formation; they later completely separate from the ganglia, and out of their remains the exsertile vesicles form. In *Pauropus*, on the other hand, the ganglia undoubtedly undergo enlargement at the expense of cells which arise from the 'ventral organs', and in the abdominal segments the remains of the 'ventral organs' are themselves finally incorporated into the ganglia; in the collum segment alone do vestiges of the 'ventral organs' remain to produce an organ comparable with the exsertile vesicles of Symphyla. In the embryo of *Scolopendra*, as shown by Heymons (1901), the ganglia arise from epidermal pits, with orientated cells recalling those of 'ventral organs', but these pits later invaginate below the surface and become part of the ganglia. Yet the developing nerve-cord of *Scolopendra* displays, in common with *Peripatus*, one important feature which has so far not been found in any other myriapod or insect embryo: in *Peripatus* the dwindling 'ventral organs', as they approach one another before fusing in the mid-line, remain connected with the widely separated ganglion-halves by a peculiar string of cells (Kennel, 1886; Sedgwick, 1888); in *Scolopendra* a similar string of cells (Mittelstrang) is present, but in this myriapod the two ganglion-halves become drawn together, and the cells of the 'Mittelstrang' remain to form a median band of neuroglia tissue within the completed ganglion. In Diplopoda, also, according to Pflugfelder (1932), the ganglia arise by invagination of pits of orientated cells; but a detailed account of the development of these ganglia has not yet been given.

The debatable question of the significance of these peculiar structures has been discussed above (Emb. Dev., section 13 (*b*) i). They are not found in insects, nor in any other arthropod hitherto examined. Their prevalence in the myriapods seems therefore to point to a closer affinity of the latter with *Peripatus*, than with any other surviving arthropod.

The brain, in its external form, displays some features unexpected in a myriapod. From the descriptions of Saint Remy (1887), Holmgren (1916), Fahlander (1938), and others, it is known that the three component ganglia

of the brain are, in most myriapods, imperfectly fused paired lobes, the antennary lobes (deutocerebrum) lying beneath the protocerebral lobes, and the tritocerebral lobes, in turn, beneath the deutocerebrum. But in *Pauropus* the deutocerebrum gives no external evidence of its originally paired condition, and is so intimately merged with the protocerebrum, that there is scarcely any external demarcation between the two. The tritocerebral ganglia are small, as in other myriapods, in which respect they differ from those of Crustacea. They remain as separate lobes, and display, moreover, one primitive feature, in that they merge below into the sub-oesophageal ganglion, without the intervention of a free connective. Such a condition is found also in some chilopods (*Scutigera, Lithobius*), but in other chilopods and in those diplopods that have been examined the tritocerebral ganglia are drawn up near the brain, thereby exposing the connectives which are free from nerve-cells. In insects, as is well known, a still greater specialization is attained, the tritocerebrum merging so closely into the contour of the brain as to become almost obscured. In one respect, however, the tritocerebrum of *Pauropus* shows a surprising specialization: the upper ends of the paired ganglia fuse above the oesophagus, with the formation of a second commissure and an unpaired connective with the frontal ganglion, there being nothing comparable with the 'stomatogastric bridge' of other myriapods.

In the deutocerebrum the presence of separate motor and sensory nerves to the antenna is noteworthy; this condition seems to be general for myriapods.

The presence of what seems to be a vestigial pre-antennary ganglion, or ganglion of the hypothetical pre-antennary segment, is also noteworthy. Such a ganglion was first observed by Heymons (1901) for the embryo of *Scolopendra*, and is known also in Symphyla (Tiegs, 1940). It is not known in insects nor in diplopods, though its discovery in the latter, and perhaps in primitive insects also, would not cause surprise.

The protocerebrum is distinguished, even among myriapods, by the relative simplicity of its structure. As in other blind members of the group, 'visual masses' are not developed. Globuli cells, also, are absent, as in Symphyla, Collembola (*Tomocerus*), and the chilopod *Geophilus*. Yet peduncles are recognizable; but, as in *Julus*, they are related to a 'medial body', and not to a corpus centrale, the latter being much reduced. A 'medial body', it may be observed, is found also in some Chilopoda, and in the Diplura, but is quite unknown in Crustacea. On the whole, the protocerebrum seems to display a simplified diplopod character; but in the absence of adequate data both for *Pauropus* and for other myriapods a more searching comparison cannot be made. In its development out of three separate ganglion masses, the protocerebrum of *Pauropus* resembles that of *Scolopendra*, Symphyla, and insects (the diplopods have not been properly examined on this point).

It is evident, from the foregoing discussion, that the brain of *Pauropus*, despite some singular features, conforms to the myriapod type. In what relation does the latter stand to that of *Peripatus*? According to Holmgren's description the brain of *Peripatus* consists of a central portion containing

the corpora pedunculata, of a pair of lateral ganglia associated with the antennae, and of a hinder part, from which the nerves to the mandibles arise. The presence of corpora pedunculata in the central portion seems to identify this region with the archicerebrum (ganglion of prostomium) of annelids, and with the more highly developed protocerebrum of myriapods and insects, of which it is probably the forerunner. On the view here adopted the lateral ganglion masses from which the nerves to the antennae originate would be the equivalent of the much-reduced pre-antennary ganglia of myriapods, i.e. ganglia of the first segment. It seems difficult to avoid this conclusion; for the first segment of *Peripatus* must have the status of a true segment and not of an acron, since it contains in the embryo a well-developed coelome, and, as all writers agree, a pair of vestigial segmental organs (Kennel, 1886; Sedgwick, 1887; Sheldon, 1888; Evans, 1901). Yet in Kennel's clear description of the developing brain the entire ganglionic mass of the first segment is shown to originate from a single pair of invaginating 'ventral organs'. Evans, indeed, later sought to demarcate an archicerebrum within the mass of developing ganglionic tissue, but the evidence for this is not clear. A renewed investigation on this point is needed; it is perhaps significant that in *Pauropus* the Anlage of the pre-antennary ganglia is at first confluent with that of the protocerebrum, and only later becomes separately defined.

The presence, both in Diplopoda and Chilopoda, of eyes consisting of aggregations of ocelli points to their probable occurrence also in the forerunners of the Pauropoda. But in all the members of this group that have so far been discovered eyes are absent, as they are also in Symphyla, Diplura, and various diplopods that habitually live in the dark. For purposes of phylogenetic discussion the chief problem concerns the compound eye. No theory which presents the Myriapoda-Insecta as a continuous line of evolution of terrestrial arthropods, and excludes the Crustacea, may ignore this remarkable organ, for there can be no denying its almost complete identity in Crustacea and higher Insecta. That the compound eye has evolved from a simple aggregation of ocelli is now generally agreed; for there is much similarity in structure of an ommatidium and an ocellus, and moreover, in the larvae of many holometabolous insects, as well as in adult fleas and Strepsiptera, the eye is found in this condition. It is only by multiplication and aggregation into a compound eye that groups of ocelli could achieve even such moderate resolution as the insect eye is capable of. Korschelt and Heider, who support the theory of a myriapod origin of insects, point out that a compound eye must have appeared in this way at least four times in arthropods, namely, in scorpions, Crustacea, *Scutigera*, and insects, the organ of insects and Crustacea being almost indistinguishable. Hesse (1901), on the other hand, regards the possibility of convergence as too remote to consider. Yet he has himself provided the evidence that the compound eye of higher insects has probably evolved within the class Insecta itself; for in *Lepisma* the compound eye is of the bilaminate type found in *Scutigera*, a vestige of this being recognizable even in the eye of *Periplaneta*. If the remarkable similarity

between the eyes of higher insects and Crustacea be accepted as evidence of affinity, then it must be of the most immediate affinity; but this is hardly compatible with the many deep-seated differences between the two groups.

Of the sense-organs of *Pauropus*, the most remarkable are the pseudoculi and the strange tactile flagella of the antennae. The latter, as Verhoeff has already observed, seem to be modifications of the peculiar clubbed 'hairs' which abound on the chitin of *Pauropus*, and seem to be quite unique. The pseudoculi are also very specialized organs, apparently unknown in other myriapods. Whether there is any affinity between them and the widely distributed organs of Tömösvary is uncertain.

EVOLUTION OF THE MYRIAPODA AND INSECTA, AND THEIR RELATION TO OTHER ARTHROPODA

Any theory of the evolution of the myriapods, and their derivatives the insects, must embrace the wider problem of their ultimate origin. But on this question the greatest perplexity still prevails, for the fossil evidence has not proved helpful, and both embryology and morphology speak with an uncertain voice.

The Myriapoda may have arisen from the Crustacea, or from the Trilobita, or from some extinct *Peripatus*-like ancestor. But it is also possible that they may have sprung from an extinct type of arthropod that cannot be assigned to any of these groups, as in Tillyard's (1930) 'Protaptera' theory.

In theories of the last-named type, in which the hypothetical ancestor is conceived to embody a combination of characters requisite to the particular case, conviction must depend ultimately on the discovery of such an ancestor as a fossil. Here the actual evidence for the existence of such an ancestor, in the form of the supposed pre-Cambrian *Proadelaidea* (David and Tillyard, 1936), seems much too meagre to justify the important conclusions that have been drawn from it. For the present, therefore, we can turn only to known forms of arthropod.

In the Crustacea we have a sharply delimited group of already markedly specialized and primarily aquatic arthropods, whose most primitive members, the Branchiopoda, seem to show evidence of near-annelid affinity. In the theories of Carpenter (1903), Börner (1909), and Crampton (1919, 1928) the higher Crustacea are conceived as the ancestors of Myriapoda and Insecta, the arguments being variously based on such external characters as structure of mandibles and of head-capsule, and the supposed identity in segment number. It is the already high specialization of the Crustacea which presents the main difficulty for these theories. In no myriapod or insect is there any true biramous appendage of the crustacean type, nor is it ever met in the embryo. The post-cephalic region of Crustacea, already differentiated into thorax and abdomen, with diversified forms of appendages, is in marked contrast to the undifferentiated post-cephalic region of myriapods, with its serially uniform walking-legs. The specialized alimentary canal, with cephalic 'stomach' and 'liver', and, in higher Crustacea, diminutive mid-gut,

is also quite different from the thoroughly simple type of gut that prevails throughout the myriapods and primitive insects, while the pronounced shortening of the heart in the higher Crustacea is never met with in any myriapod or insect, though in the more primitive Crustacea an elongate heart of the myriapod-insect type is found. The remarkable ectodermal and mesodermal teloblasts of crustacean embryos are never encountered in the ontogeny of myriapods or insects, while the marked suppression of coelomic sacs is in contrast to their strong development in most myriapods and primitive insects. There is, moreover, never an indication of a nauplius phase in the development of any myriapod or insect, yet it is of universal occurrence in Crustacea. In thus rejecting the crustacean theory one must, however, fairly face the evidence from the compound eye; this question has already been discussed, the resemblance being attributed to convergence. The retention of segmental organs in the head of Crustacea and some myriapods is also difficult to assess; it is noteworthy that in Crustacea they are excretory organs and in myriapods salivary glands. The problem of the mandibles, which Börner in particular has stressed, does not seem a serious one, for similar feeding habits may well be expected to engender similar feeding organs.

The Trilobita are the most primitive and generalized of all known Arthropoda, unless *Peripatus* be admitted to the phylum. Formerly regarded as Arachnida, they are now, following the discovery of their biramous appendages, usually ranked with the Crustacea. Of their internal anatomy nothing is known beyond the fact that they had a straight intestine with cephalic stomach and probably liver (Raymond, 1920), features which they share with Arachnida and Crustacea, but not with myriapods and insects. The appendages were, except for the simple antennules, serially similar biramous structures, there being four such appendages on the cephalon. The terms 'antennae', 'mandibles', &c., that are sometimes applied to the cephalic appendages cannot therefore be used with strict propriety. In the most recent work of Walcott (1918, 1921) the trilobite appendage is described as consisting of a coxopodite (always with gnathobase), an endopodite (walking-leg), an exopodite (which may be a broad setiferous swimming (?) blade or a spiral filament fringed with delicate branchiae), and a blade-like epipodite.[1] Since chelicerae are absent, the Trilobita cannot be Arachnida. But there are also strong grounds for excluding them from the Crustacea: (*a*) from their appearance early in the Cambrian to their extinction in the Permian, they preserve a remarkably stereotyped body-form, differing from that of any known crustacean; (*b*) there is no diversity of appendages, which is so characteristic of Crustacea; (*c*) the larva in all known cases is a 'protaspis' and not a crustacean nauplius. Yet in the wonderful mid-Cambrian marine fauna discovered by Walcott the trilobite affinities of the Crustacea are clearly brought out. *Burgessia*, *Waptia*, and *Yohoia* were Branchiopoda, but,

[1] Störmer (1933), who has since re-examined Walcott's material, agrees with Raymond that there is no real evidence of an epipodite.

as Raymond (1920)[1] has shown, with strong evidence of trilobite affinity
in their appendages, and this is especially clear in the later restorations given
by Walcott (1931); particularly noteworthy are the mouth-appendages,
which were walking-legs without differentiation into mandibles and maxillae.
In the remarkable *Cheloniellon* described by Broili (1933) we have evidence
for the survival of such primitive Crustacea even into the lower Devonian.
Yet this can hardly be construed as valid evidence for the derivation of these
primitive Crustacea from trilobites. Of the long pre-Cambrian ancestry of
the trilobites from annelids nothing whatever is known; but we may presume
that it was from some pro-Trilobite Arthropod that the primitive Crustacea,
with their more worm-like body, arose. The affinity between Trilobita and
Arachnida seems to be even closer than with Crustacea. Whether the
'trilobite larva' of *Limulus*, with its chelate appendages and unsegmented
abdomen, but otherwise very trilobite-like body, implies such affinity may
perhaps be doubted. On the other hand, there seems to be good evidence
for a 'protaspis' stage in the embryo of *Limulus* (Iwanoff, 1933). The recent
work of Raasch (1939) on the Aglaspida has brought out the merestome nature
of these Cambrian arthropods, for in one genus chelicerae have been revealed;
yet, except for their long articulated telson spine, they show a most remarkable
resemblance to trilobites, and especially is this the case for the mid-Cambrian
Beckwithia described by Resser (1931). This would seem to imply the former
existence of even more primitive types derived either from the trilobites or
from the base of the trilobite stem. Their distinguishing features would be
a general resemblance to trilobites, the presence of trilobite appendages
(including simple antennules), and the occurrence of an articulated telson
spine. It is quite possible that the remarkable mid-Cambrian genera
Molaria, *Habelia*, and *Emeraldella* described by Walcott (1912), but referred
by him to the merestomes, fulfil these conditions.

From the presence of chelicerae and uniramous appendages in Aglaspida,
Raasch has argued against any close affinity between them and trilobites.
Exopodites are, however, notoriously difficult to detect except in the most
perfect material; in any case, the appendages of the mesosoma of *Limulus*
are biramous. Moreover, Störmer (1933), who has re-examined the remark-
able trilobite material of Beecher, Raymond, and Walcott, declares that in
Limulus 'the exopodite is much more like that of the trilobite than is that of
any Crustacean'.

Such being the affinities of the Trilobita, is there any reason to suppose,
as Handlirsch (1925) and Raymond (1920) do, that the Myriapoda may have
arisen from them? The already stereotyped body-form of the Trilobita is in
marked contrast to its simplicity in the generalized myriapods, and through-
out the whole range of known trilobites there is not a single form that shows

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[1] In a more recent paper Raymond (*Bull. Mus. Comp. Zool. Harvard*, 1935, **76**, no. 7)
considers it necessary 'to remove the Mid-Cambrian forms from the sub-class Branchiopoda,
although still considering them as belonging to the stock from which the modern groups
were derived'.

even a passing resemblance to a known myriapod. This may perhaps be attributed to the adoption of a terrestrial habitat by the latter; but terrestrial adaptation in other groups of arthropods has not so completely obscured all trace of affinity with related aquatic groups (e.g. scorpions and their aquatic Silurian ancestors), and even the most ancient myriapods, the Silurian and Devonian *Archidesmus* (Peach, 1882, 1889), give no sign of it. It must however be acknowledged that the nature and diversity of the terrestrial fauna of the Silurian and Ordovician are almost completely unknown, and that some fortunate discovery, such as the Rynie chert of a later period, may yet reveal an unexpected terrestrial myriapod fauna with trilobite affinities. Yet, on the whole, the evidence suggests that this will not be the case: neither in the adult nor embryonic condition of any myriapod do we meet with appendages of the trilobite type, the supposed biramous character of the head-appendages of chilopods (Tothill, 1916) being a misconception. The simple alimentary canal of myriapods is also in contrast to that of trilobites, with its cephalic 'stomach' and digestive glands of the crustacean type; nor do we ever encounter in myriapods, as we do in *Limulus*, an embryonic phase comparable to the 'protaspis' of trilobites. The embryonic development of trilobites can never be known; but if that of *Limulus* may serve as a guide, it would seem that nerve-ganglia developed without the formation of 'ventral organs', which are so characteristic of primitive myriapods.

The possibility of seeing in some ancient *Peripatus*-like form the actual ancestor of myriapods and insects is no new idea. It is stated, for example, by Sedgwick (1909) in the following words, which seem to express most clearly the real affinities of these terrestrial arthropods: 'The classes Insecta, Onychophora and Myriapoda are the survival of a once great and continuous group of land Arthropods, a large number of which have become extinct, leaving two groups, Insecta and Onychophora, each fairly compact and showing but little variation of organization, and one, the Myriapoda, loose and heterogeneous, and with considerable gaps between the orders.'

Not the least surprising of the many remarkable features of *Peripatus* is the fact that it is terrestrial, even though it stands nearer to the annelids than do any of the true Arthropoda. But the mid-Cambrian *Peripatus*-like *Aysheaia* (Walcott, 1911*b*, 1931) is found in association with the remains of marine animals, and was therefore itself of marine or at least littoral habitat. But even the recent *Peripatus* gives ample evidence of primitive adaptation to its terrestrial environment: one might point to its low capacity to resist desiccation; to its retention of ineffective 'nephridia', whose use as excretory organs would entail a drain on its water economy (Manton, 1937); to the crude device for achieving internal fertilization, consistent with a terrestrial habitat (Manton, 1938); and finally, to the widespread prevalence of viviparity as a means of protecting the eggs not yet fully adapted to the land. Has this migration to the land been without further issue, a kind of evolutionary cul-de-sac? Or has it set in train the evolution of the great terrestrial groups of myriapods and insects? Should the latter be

the case, then it will have been one of the most momentous events in the whole Palaeozoic age.

In the general form of the body and its appendages, *Peripatus* is devoid of those specializations which distinguish the Trilobita and Crustacea. Except for the presence of a rudimentary head, composed of the three most anterior segments, there is no demarcation of the body into regions. The alimentary canal, quite unlike that of Crustacea, is simple and closely resembles that of myriapods, and its mid-gut epithelium has, as in some myriapods, an excretory function. The heart also is similar to that of myriapods, and quite unlike that of higher Crustacea. In the embryo a succession of strongly developed coelomic vesicles develops, vestiges of which persist in the segmental organs; in myriapods and primitive insects, also, the coelomic vesicles are strongly developed, and there is a recurring tendency for authors to compare them with those of *Peripatus* and not of Crustacea. Segmental excretory organs, it is true, do not develop in myriapods and primitive insects, but there is often a marked development of coelomoducts in the embryo, and these rudiments are not met in Crustacea. The pre-mandibular salivary gland of *Pauropus* and Symphyla is a derivative of such a segmental organ, and it is probably no mere coincidence that the salivary gland of *Peripatus* is the segmental organ of the same (third) segment. The segmental organs of the penultimate segment of *Peripatus* become the exit ducts for the gonads; this holds also for the opisthogoneate myriapods, and there is reason to believe that, for myriapods, this is the more primitive condition, since in the opisthogoneate forms alone do the gonoducts develop out of embryonic coelomoducts. Finally, the nerve-ganglia of *Peripatus* develop in association with 'ventral organs', and these remarkable structures are also present in myriapods but in no other arthropod (the supposed 'ventral organs' of Pycnogonida are, according to Dogiel (1913), not comparable with those of *Peripatus*).

These facts, unless they are quite misleading, seem to point to the Onychophora as the not very distant progenitors of Myriapoda. The evolution of the latter must have taken place upon a background of increasing adaptation to the terrestrial environment, and this must have involved not only the freely living animals, but also their eggs.

The evolution of the 'closed box' type of terrestrial egg (cleidoic egg of Needham, 1931) presents special problems, to evade which the device of viviparity may, as in *Peripatus*, have been temporarily resorted to. In its most highly developed form (in birds) the cleidoic egg contains its own water requirements, and relies on the environment only for its supply of oxygen. In most myriapods and primitive insects this degree of independence has not been attained; the yolk is inadequately supplied with water, which must be supplemented by absorption from without, and oviposition is therefore restricted to damp localities. Although remarkable adaptations to dry conditions may at times have been evolved (e.g. *Sminthurus*), there seem to be few exceptions to the rule that the eggs depend for their development on an

external supply of water. The developing embryo therefore soon outgrows the available space within the chorion, which is stretched and usually ruptured. This is probably the explanation of the widespread occurrence of pupoid phases in myriapods. In many higher insects, also, swelling of the egg occurs as development proceeds (see review by Buxton, 1932), and special water-absorbing organs, as in *Melanoplus* (Slifer, 1938), may be present. Yet in its most perfected form, the land-adapted insect egg is often found, encased in a thick, firm, and indistensible chorion, exposed to the most adverse climatic conditions. The nature of these adaptations does not seem to have been adequately investigated.

Upon leaving the egg, water conservation remains one of the chief problems of the small terrestrial animal. The myriapods have largely evaded this problem by restricting their habitat to moist localities. Most small myriapods and primitive insects desiccate rapidly in dry air, and only occasionally do we meet well-adapted land forms, such as *Scutigera* and *Lepisma*. Early in the evolution of the myriapods, we may suspect, an increasing impermeability of the chitin to water developed; but physiological studies on water-conservation in myriapods have lagged far behind the illuminating work on insects. As the external chitin of the soft-bodied *Peripatus*-like animal acquired greater rigidity, this must have led to an accentuation of the body-segments, and the jointing of appendages. To compensate for the loss of flexibility there was 'developed a mechanism of telescopic movement between successive body segments, by the simple device of retaining non-sclerotized areas in the posterior parts of the primitive segments, thus establishing a secondary segmentation in which the longitudinal muscles become intersegmental instead of intrasegmental in action' (Snodgrass, 1938). The hardened exoskeleton must, in its turn, have produced the conditions for the development of rapidly moving striated muscle, and for the eventual evolution of those mechanical contrivances to which the arthropods owe their immeasurable superiority over the annelids.

Except for the simplicity of its head, such an animal would differ but little, in external appearance, from a generalized myriapod. The myriapod head-capsule may be presumed to have arisen by the incorporation of two, and later a third abdominal segment, into the primitive head, the latter having become stabilized as the three-segmented procephalon of myriapod-insect embryos. (This would necessarily entail the conversion of the mandibular appendage of the *Peripatus*-like ancestor into an antenna. It is not necessary to discuss the possible difficulty that might arise from the transformation of the mandibles, i.e. enlarged claws, of a recent *Peripatus* into antennae, since in the only Palaezoic onychophoran of which we have any information (*Aysheaia*, see Hutchinson, 1930), the 'mandibular' appendage is an undifferentiated ambulatory leg, no true jaws being present.) *Pauropus* is to be looked upon as a dwarfed and simplified, but also in some respects very specialized, survivor of a primitive stock of myriapods, in which only two such segments have been added to the procephalon (DIGNATHA); and it is

probable that, when their development is better known, the diplopods also will be found to be members of this group, though specialization in another direction, with the formation of diplosegments, has here taken place. The presence of a limbless collum segment in Diplopoda and Pauropoda even suggests a community of origin for these two groups of Dignatha.

With the incorporation of the second maxillary segment into the head there arose the TRIGNATHA. These comprise the Chilopoda, Symphyla, and Insecta. Within the Trignatha, by further specialization, arose the LABIATA, in which the second maxillae fused to form a new lower lip to the pre-oral cavity, within which the first maxillae operated, the lower lip of the Dignatha apparently remaining as 'hypopharynx'. They comprise the Symphyla and Insecta. Their great antiquity is attested by the recent discovery of Collembola in the middle Devonian (Tillyard, 1928; Scourfield, 1940).

This classification, based on the structure of the head-capsule, does not necessarily imply any close affinity between chilopods and insects. The chilopods remain, indeed, a puzzling group. In their early development they display features reminiscent of *Peripatus*. The mesodermal somites are also extremely generalized, in one respect even more so than in *Peripatus*, for both dorsal and ventral blood-vessels arise by apposition of the somitic walls, as in annelids. In the development of the nerve-cord, as described by Heymons, there is a marked resemblance to *Peripatus*, and this applies also to the development of the gonads. On the other hand, the conversion of the first pair of abdominal legs into poison jaws is a specialization which we find in no other myriapod. Their fossil record is not helpful: whereas diplopod remains are abundant in Carboniferous and later formations, there are only a few disputed chilopod remains earlier than the Tertiary, Scudder (1890) having attributed to the Chilopoda some rather nondescript remains from the Carboniferous of North America.

Of all the Myriapoda the Symphyla, being alone Labiata, must stand nearest the insects. In this connexion the Collembola present a special problem; for while, among insects, *Campodea* and its allies seem to be the most nearly related to the Symphyla, it is actually in the Collembola that myriapod features to the greatest extent prevail. Their early developmental processes are quite non-insectan in character, and are of the Diplopoda-Symphyla type. The reproductive organs are, except for the terminal genital opening, also quite unlike those of any insect, and resemble those of Diplopoda and Symphyla. The visual organs are aggregates of ocelli; such ocelli are characteristic of myriapods, and occur only rarely in adult insects, and then probably in consequence of degeneration. Organs of Tömösvary, prevalent in the Myriapoda, are found also in Collembola, but not in true insects. They differ, moreover, from all insects, by the presence of never more than nine post-cephalic segments in the embryo. The presence of three pairs of walking-legs is, on the other hand, an insectan character; but as evidence for their inclusion in the Hexapoda this is offset by the presence of two more abdominal appendages (in addition to the 'ventral tube'), which also play

an important role in their locomotion. Minor insectan characters, which are, however, difficult to evaluate, are the unsegmented mandibles, and the development of the ganglion cells of the embryo from 'neuroblasts' (Claypole, 1898; Philiptschenko, 1912), which are general throughout insects, but quite unknown in myriapods. As Imms (1936) has convincingly shown, there can be little ground for including Collembola among the insects; the available evidence is consistent with the hypothesis that they constitute an independent group within the Labiata, in which anamorphosis has been suppressed, and in which consequently a progoneate condition has not supervened, and that specialization has taken the form of elaboration of the springing apparatus.

It is not unlikely that when the development of the Protura becomes known, they will be found to form a fourth group within the Labiata.

From time to time remains of ancient and extinct myriapods have been discovered which cannot be accommodated within any of the existing groups: *Archidesmus* (Silurian, Devonian), *Kampecaris* (Devonian), Peach, 1882, 1889; *Palaeocampa* (Carboniferous), Meek and Worthen, 1865, Scudder, 1884; *Latzelia* (Carboniferous), Scudder, 1890. In these scanty remains we have the only real evidence of a former line of myriapod descent, from which the present rather isolated groups may be supposed separately to have arisen. The state of preservation does not, however, suffice to determine the all-important character of the head-capsule. In particular, there is no known example of the hypothetical MONOGNATHA, which may be supposed to have bridged the gap between the Onychophora and the Dignatha.

The scheme of affinities which the foregoing discussion suggests may be put thus:
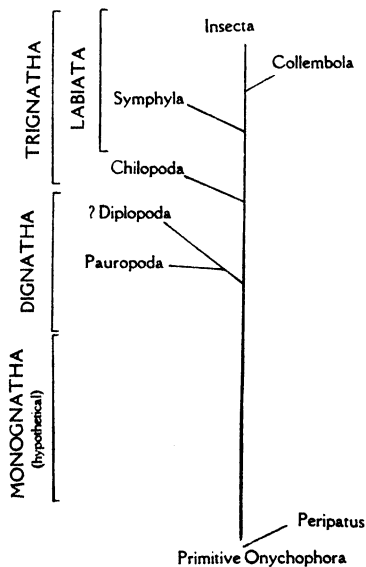
The Myriapoda, we may suppose, were, like the Onychophora, primarily opisthogoneate, for in the opisthogoneate groups alone do the gonoducts develop out of coelomoducts in the embryo. From the prevalence of ana-morphosis in recent myriapods we may suspect that some form of ana-morphosis, the consequence of deficient yolk in the egg, was prevalent also in primitive myriapods; but whenever conditions favoured the development of the reproductive ducts before the completion of anamorphosis, it must have led to the formation of a new gonopore, remote from the zone of growth. Thus arose the progoneate groups. (Even in insects secondary genital openings, remote from the terminal genital opening, may at times appear (cf. Imms, 1936).)

We may further suppose that, in adaptation to a terrestrial existence, the segmental organs were soon discarded, for already in *Peripatus* they have been functionally superseded as excretory organs by the mid-gut epithelium, and only in the head were some retained as salivary glands. It is probable that the mid-gut epithelium, as well as fat-body, which seems to have arisen by retention of embryonic vitellophages, assumed the role of excretory organs. But from the presence of Malpighian tubes throughout the Myriapoda it would seem that these organs soon arose to meet the special needs of an excretion consistent with a stringent water economy. Yet it is surprising to see how often these organs have again been discarded, as effective excretory organs (*Pauropus*, Collembola, Symphyla, *Japyx*, *Campodea*), in favour of excretion by the fat-body or mid-gut wall.

Terrestrial adaptation of the respiratory organs involved the development of the tracheal system. Tracheae are present in such diverse forms in different myriapods and insects that a polyphyletic origin is commonly ascribed to them; yet it is difficult to concede that an effective tracheal system, like that of most chilopods, should be completely discarded in favour of a wholly new system, as in *Scutigera*. It seems more likely that there was evolved a general tendency to develop tracheae, and that, in consequence of mutation, the development of these tracheae became localized in different areas of epidermis. The evolution of tracheae was of the greatest importance, not only in pro-moting enlargement of the body, but in resolving the dilemma between the need for a cutaneous respiration and the inevitable water-loss through a thin chitin that it entailed; for in the better land-adapted types the investing chitin becomes increasingly impermeable to water, while evaporation through the tracheae is subject to control by temporary closure of the spiracles. It is noteworthy that among Collembola *Sminthurus*, which alone possesses tracheae, is alone resistant to desiccation.

The greater freedom of movement that had its origin in the development of mechanical devices in the hardening exoskeleton must have profoundly affected, and in turn been conditioned by, the elaboration of the nervous system and sense-organs. For swiftly moving animals the tactile and olfactory senses must become subordinated to the visual sense; and accordingly we find, in passing from the myriapods to the insects, a great relative increase

in the visual centres of the brain, and a retrogression in the olfactory centres (cf. Hanström, 1928). Amongst myriapods the eyes have remained mere aggregates of ocelli, and only in the swiftly moving *Scutigera* have large compound eyes developed. In insects the conversion of these aggregates of ocelli into compound eyes, with their enhanced resolving power, is almost certainly correlated with their greater freedom and speed of movement.

In thus deriving the Myriapoda directly from some ancient terrestrial *Peripatus*-like ancestor, and excluding from the line of descent the great Trilobite-Crustacean-Arachnid branch of the phylum, it is possible, though not inevitable, that we are committed to a polyphyletic origin of the Arthropoda. Many years ago this very question was discussed by a group of writers, with singular lack of unanimity (see Hutton and others, 1897); yet so distinguished a student of arthropod morphology as Lankester could still declare (1904) that it was 'impossible to conceive of them as having a polyphyletic origin'. That the development of heavy plating in the outer chitin, necessitating the production of segmented appendages and of striated muscle, might arise repeatedly as a specialization in soft-bodied annelids, could be conceded; but there are other basic features of arthropod structure—the haemocoele and ostiate heart, the appendicular jaws, and the cephalization of segments—for which this might less readily be granted, and these features are all displayed, in some measure, by *Peripatus*. A haemocoele is, however, not a unique character restricted to arthropods, for it is encountered also in Mollusca and in some annelids, and the jaws of arthropods are gnathobases, while those of *Peripatus* are enlarged claws, and 'the whole musculature and movement of the jaws ... contrasts absolutely with the Myriapoda, Crustacea and Insecta' (Manton, 1937). It is therefore possible that, if the fossil record ever discloses the ancestry of Crustacea and Trilobita, it may reveal a descent from annelid ancestors quite unlike the Onychophora. The enormous assemblage of animals embraced by Cuvier's ARTICULATA will then comprise a single phylum, distinguished from the other phyla by its own peculiar plan of organization; and within this great phylum there will have arisen, by specialization, and with much convergence, two separate lines of descent, of which one is constituted by the Crustacea, Trilobita, and Arachnida, the other by the Myriapoda and Insecta.

SUMMARY

1. The minute egg is heavily yolked, and is devoid of periplasm or vitelline membrane. Shortly before laying, the germinal vesicle phase gives way to one in which the chromosomes reappear as thirteen 'tetrads' in a central clump of cytoplasm. The latter moves to the periphery, and in this first meiotic prophase the egg is laid. The 'polar bodies' do not separate from the egg. In most eggs they degenerate very rapidly. Male and female pro-nuclei fuse in the centre of the egg to form a resting nucleus.

2. Cleavage is total and unsynchronized, the cleavage-cells becoming arranged as yolk-pyramids around a central blastocoele. This blastula per-

sists up to about the eighty-cell stage, when a gastrula is formed by migration of one, or at most two cells, from the layer of yolk-pyramids into the blastocoele to form the endoderm.

3. The gastrula is succeeded by a blastoderm of the familiar myriapod-insect type; the cleavage nuclei, except those of the endoderm, move into the peripheral layer of accumulating cytoplasm, while the internal cell-partitions break down. Total cleavage is thereby replaced by a superficial cleavage within the blastoderm. A blastodermic cuticle is secreted.

4. Out of the blastoderm there now differentiates the germ-band; it is of the usual elongate myriapod type, and extends over the anterior and posterior poles of the egg on to its upper half. At no time does a ventral flexure form. The rest of the blastoderm becomes much thinned out and is a provisional body-wall.

5. From the inner surface of the blastoderm isolated cells with enlarging nuclei have meantime migrated as yolk-cells into the yolk; from them will eventually form the fat-body of the larva.

6. During the development of the germ-band the latter becomes the source of the developing mesoderm, cells separating from it in great numbers along its length, and so coming to form a second, and at first very irregular layer, internal to the ectoderm.

7. The early developmental processes are of the usual myriapod type, except for the presence of an easily recognized gastrula. The survival of the latter is of importance for the interpretation of the specialized myriapod-insect type of ontogeny; in particular, the blastoderm phase is found to be a post-gastrula stage, and not a blastula as commonly believed.

8. Stomodaeum and proctodaeum are the first structures to appear in the germ-band. The formation of the head-lobes soon follows. Then the Anlagen of the antennae arise, being at first post-oral in position; and after them appear the Anlagen of the mandibles and maxillae. The premandibular segment does not bear even the rudiments of appendages. The segment behind the maxillary segment is the collum segment, without appendage-Anlagen, and it remains part of the abdomen; there is, therefore, no second maxillary segment. The Anlagen of the first, second, and third legs then appear in succession. Intersegmental lines form only in the advanced embryo; when these eventually appear, they reveal two segments behind the fourth abdominal (third leg-bearing), namely the fifth abdominal and anal segments. The fifth segment of the embryo becomes the fifth of the adult animal, so that the teloblastic formation of new segments must proceed by the budding off of new segments from the stationary anal segment.

9. The embryo now slowly swells, presumably owing to absorption of water, and so outgrows the available space within the egg. A gradually enlarging rent appears in the egg-shell, and after several days the embryo emerges from it in a quiescent 'pupoid' phase. Unlike the 'pupa' of other myriapods, it shows but little resemblance to the future larva. Within the pupa the first teloblastic segment (sixth abdominal) appears.

10. The development of the abdominal segments, both in the embryo and in the pupa and larva, shows that there are no 'diplosegments' in *Pauropus*; the tergites are derivatives of simple segments, and the apparent 'diplopody' arises from the presence of a wedge-shaped segment, with reduced tergal wall, behind the tergite-bearing segments.

11. The differentiation of the head-capsule out of the primitive head-segments is attended by the familiar inturning of the sternal wall of the post-oral segments to form the floor of the pre-oral cavity; and by the curving forward of the more lateral parts of the segments towards the front of the head. The antennae are thereby carried into a completely pre-oral position. The pre-mandibular epidermis becomes rolled under to form the roof of the pre-oral cavity (inferior surface of clypeus).

12. The head is composed of a procephalon, in which pre-antennary, antennary, and pre-mandibular segments are represented; and of a gnatho-cephalon, consisting of only two segments, the mandibular and maxillary. Superlinguae, derived from the mandibular epidermis, are present. The mandibles are closed in by the inturned margins of the clypeus; they are unsegmented and adapted to a diet of semi-fluid food. The maxillae display a cardo, a stipes, and a lacinia. Between them is the intermaxillary plate (sternite of maxillary segment).

13. At no time does the germ-band display a ventral flexure. Embryonic membranes are absent. An embryonic 'dorsal organ' appears, but it is not of the type found in Symphyla, Collembola, and *Campodea*.

14. In the early germ-band the mesoderm tends to aggregate laterally into two bands of cells, from which the succession of somites arises. Al-though many of the somites soon display very small coelomic cavities, they remain poorly developed, and coelomoducts do not appear. Since there are no blood-vessels in *Pauropus*, they do not contain any vasoblasts; they are, more-over, quite unique in that they do not even contribute any mesoderm to the mid-gut wall, the splanchnic mesoderm arising entirely from the mesoderm of the stomodaeum. Nor do they participate in the formation of the genital rudiment. The dorso-lateral muscles also are not derived from the somites.

15. Between the rows of somites is a layer of unsegmented 'median meso-derm'; out of it develops the genital tube, as well as a median band of neuro-glia (?) tissue in the nerve-cord.

16. Although the somites are diminutive, a complete set is present. They are the pre-antennary (vestigial), antennary, pre-mandibular, mandibular, maxillary, collum, second, third, and fourth abdominal somites; a fifth abdominal and very small anal somite form in the more advanced embryo. In addition to these there is also a small clump of 'teloblastic mesoderm' arising in the late embryo from the mesoderm that is heaped up in front of the proctodaeum; from it is generated the mesoderm of the larva.

17. From the pre-antennary somite arise the buccal dilator muscles; from the antennary and mandibular somites arise the muscles of the antennae and mandibles respectively.

18. From the pre-mandibular somite arises the large pre-mandibular gland; it opens to the side of the mandibles and is evidently a salivary gland. Its duct seems to be of ectodermal origin. There is no associated 'end-sac'. In Symphyla a pre-mandibular gland is present up to the time the larva leaves the egg, when it degenerates, leaving only its nephrocytes; in *Pauropus* alone among myriapods is it known to survive, though vestiges of it are found both in chilopods ('lymphoid tissue') and insects (sub-oesophageal bodies).

19. From the maxillary somite there develops, in addition to the muscles of the maxilla, the maxillary gland. The latter has an 'end-sac', which displays nephrocytic action to trypan blue injected into the blood.

20. The somites of the collum and other abdominal segments, as well as the anal segment, give origin to nothing but myoblasts from which most of the muscles of the respective segments develop.

21. In addition to the glands already referred to, there are present: (*a*) clypeal glands, that arise from the epidermis of the clypeus; (*b*) pseudocular glands, lying adjacent to the pseudoculi, and derived from the epithelium of the latter; (*c*) large intermaxillary glands, derived from the maxillary sternum.

22. The mid-gut epithelium is formed from the endoderm of the gastrula, the cells gradually losing their yolk and slowly increasing in number. The mesoderm of the mid-gut is derived from the mesoderm that is heaped up along the stomodaeum, whence it spreads back as an arching roof to the endoderm, the immediately underlying cells of which become arranged into an epithelium, within which excretory concretions, similar to those of the adult mid-gut, soon appear. The floor of the mid-gut remains for long free from any mesoderm; from it develops a ventral band of enlarged mid-gut cells, permanently free from excretory concretions. The hindermost tip of the mid-gut is of proctodaeal origin. The lumen of the mid-gut does not communicate with those of the stomodaeum and proctodaeum till shortly before the larva emerges.

23. The two Malpighian tubes arise from the anterior tip of the proctodaeum; they do not seem to be functional excretory tubes, since in the growing larva they begin to display a markedly degenerate character.

24. The genital rudiment does not arise out of the somites, but from the 'median mesoderm' of the fifth abdominal segment, i.e. from the vestige of the mesoderm that remains in front of the proctodaeum after the fifth somites and teloblastic mesoderm have separated from it. Embedded in it is a single primordial germ-cell. A string of cells spreads forward from this mesoderm along the roof of the nerve-cord into the third abdominal segment, thus forming the genital rudiment.

25. The fat-body does not develop out of the somites, as in insects and chilopods, but out of the embryonic yolk-cells, as in Symphyla. It is phagocytic towards injected Indian ink. The haemocoele arises from spaces left by shrinkage and withdrawal of fat-body.

26. The ganglia of the nerve-cord develop in association with 'ventral organs', of which there is a single pair in each segment, except the anal segment. In the abdominal segments the 'ventral organs' become incorporated into the ganglia. The 'median mesoderm' plays an unusual role, in that it gives origin to a median band of neuroglia (?) cells, within the chain of ganglia; there is no incorporation of 'median cord' ectoderm into the ganglia.

27. The ventral nerve-cord of the embryo comprises a mandibular and maxillary ganglion (fused into one), followed, in the abdomen, by the ganglia of the collum, second, third, and fourth abdominal (leg-bearing) segments, fifth segment, and a vestigial anal ganglion. The ganglion of the fifth, or penultimate segment, is in reality a teloblastic ganglion, which enlarges, and from which the fifth ganglion proper is separated off in the pupa, the rest remaining as teloblastic ganglion, from which, in the larva, the sixth and remaining ganglia in turn arise.

28. The brain develops out of: (*a*) a trilobed protocerebral Anlage, whose posterior lobes invaginate below the surface, but whose lateral and frontal lobes do not invaginate but display 'ventral organ' cell-disposition; (*b*) a pair of diminutive pre-antennary (?) ganglia, which give origin to a definite part of the brain between the protocerebrum and deutocerebrum; (*c*) a pair of antennary ganglia, from which the deutocerebrum will arise; (*d*) a pair of pre-mandibular ganglia, from which will form the tritocerebrum. The last three all develop in association with 'ventral organs'.

29. A detailed account of the morphology of the brain and of the cerebral nerves is given.

30. The visceral nervous system consists of: (*a*) a frontal ganglion, derived from the roof of the oesophagus; (*b*) a pair of 'oesophageal ganglia' (?), which develop in a most unexpected way, for they arise from the inner ends of the mandibular apodemes; (*c*) a stomachic ganglion, formed from the hinder end of the oesophagus; (*d*) a 'caudal' visceral ganglion, which is apparently the hind end of the last abdominal ganglion.

31. The structure and development of certain epidermal organs (trichobothria, basal antennal sense organs, pseudoculi) is described. The supposed vestigial appendages of the collum segment are not appendages at all, but seem to be related to exsertile vesicles; they develop from vestiges of the 'ventral organs' of the collum segment. The hypopharyngeal apophyses are epidermal ingrowths that arise a little in front of, and median to, the mandibles.

32. The greater part of the muscular system is derived from cells that are set free by the break-down of the somites. The dorsal longitudinal muscles are exceptional.

33. Four larval instars precede the adult animal with 9 leg-pairs. These instars have 3, 5, 6, and 8 leg-pairs respectively. The adult animal does not moult.

34. The new segments arise by proliferation of epidermal cells within the anal segment.

35. The mesoderm of the growing zone is generated entirely from the 'teloblastic mesoderm', located in the anal segment, successive clumps of mesoderm being allotted to the new segments as these successively develop. The ventral longitudinal muscles always develop precociously. Somites do not form in the mesoderm of the new segments.

36. There is no periodic renovation of mid-gut epithelium in the growing larva, such as is encountered frequently in insects; nevertheless there is considerable cell-proliferation, both in the mid-gut and in the fore- and hind-gut. The Malpighian tubes gradually degenerate, but do not disappear.

37. The fat-body of the larva is supplemented by new fat-body that develops unexpectedly from the epidermis.

38. In the newly emerged larva the genital rudiment, still sexually indeterminate, consists of a narrow string of cells lying between the intestine and the nerve-cord, and extending forward just into the third abdominal segment. Posteriorly it merges with the 'median mesoderm' of the growing zone. Its further development in the larva involves (a) slow multiplication of the primordial germ-cells located at its hinder end; (b) posterior elongation of the genital rudiment at the expense of 'median mesoderm' cells in the growing zone; (c) thickening of the anterior part of the genital tube. The exit ducts arise as a pair of epidermal ingrowths just behind the second legs, which grow round the nerve-cord and join the anterior tip of the genital rudiment. In the female one only survives, and forms the oviduct and receptaculum seminis; in the male both are retained as ejaculatory ducts. The ovary remains in its primitive position below the intestine, the oocytes arising from laterally placed germ-cells in the median germarium; the anterior end of the original genital rudiment survives in the female as the 'ductus glandularis'. In the male the gonad-Anlage bends up dorsally to either side of the mid-gut, and divides into four testes; the anterior end of the genital rudiment then splits longitudinally into the four vesiculae seminales. In fourth instar larvae the reproductive organs are often almost mature, but there is no evidence for precocious sexual functioning.

39. The classification of Myriapoda into Progoneata and Opisthogoneata does not seem to reflect the real affinities of the component groups, and in particular it fails badly for the Symphyla which are undoubtedly closely related to the Insecta. In Pauropoda and Symphyla the exit ducts from the gonads are not surviving coelomoducts, as assumed, but epidermal ingrowths, new gonopores having apparently arisen, in adaptation to anamorphosis, remote from the zone of growth. A new classification is proposed, based on the degree of cephalization and specialization of originally abdominal segments: the lowest grade of surviving myriapods are the DIGNATHA (Pauropoda and probably Diplopoda); the Chilopoda, Symphyla, Collembola, and Insecta are TRIGNATHA, and of these the Symphyla, Collembola, and Insecta are united as LABIATA by the common possession of a labium. The Myriapoda seem to have arisen, independently of the other great groups of Arthropoda, from some ancient stock of *Peripatus*-like ancestors.

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DESCRIPTION OF PLATES

PLATE X

Fig. 116, A and B. Drawings of two immediately adjacent sections (A the more anterior) from an embryo a little more advanced than that shown in the previous figure; in A only part of the section is drawn. The plane of section is only approximately frontal, the right half being a little posterior to the left.

A. The section shows the neuropilem passing between protocerebrum, deutocerebrum, and tritocerebrum and mandibular ganglion; note the pre-antennary ganglion lying median to the neuropilem. Below the oesophagus the inferior tritocerebral commissure has formed. Between the tritocerebrum and mandibular ganglion the hypopharyngeal apophysis may be seen, from which a string of cells is growing up along the side of the brain (ascending arm of apophysis). To the right side of the mandibular ganglion is the transected hind end of the mandibular apodeme.

B. On the left the section passes along the mandibular apodeme; on the right the hindermost end of the apodeme is seen. A continuous band of cells now unites them, and out of this will form the oesophageal ganglia. The deutocerebrum and a fragment of the tritocerebrum intrude from in front into the section. The protocerebral commissure is forming. The hindermost ends of the hypopharyngeal apophyses appear in the section; they have become connected with mesoderm from the oesophagus, thereby initiating the formation of the oesophageal dilator muscles. On the left side is seen part of the pre-mandibular gland. × 780.

Fig. 117. Drawing of a section approximately similar to that shown in Fig. 116 B, but from a still later embryo. The section is taken a little anterior to the protocerebral commissure,

and shows the developing septal (pre-antennary) ganglia, with the epidermal septum growing down between them. The oesophageal ganglia are now becoming recognizable, but are still connected with the tips of the mandibular apodemes. A connexion has been established between the hypopharyngeal apophyses and the mandibular apodemes (median ligament of latter). A portion of the maxillary gland with end-sac is present in the section. The two round clumps of cells just median to the hypopharyngeal apophyses are fragments of trito-cerebrum that intrude from in front into the section (cf. Fig. 116 B), and must not be confused with the oesophageal ganglia. × 780.

Fig. 118. Drawing of part of a section through anterior end of a very advanced embryo, to show the oesophageal ganglia. The latter have become completely detached from the mandibular apodemes (present in the immediately preceding section), but have not yet become associated with the oesophagus. × 780.

Fig. 119. Approximately 'horizontal' section through head of a young pupa. The asymmetry of the section is illustrated by the difference in the mandibles, and the almost complete absence of clypeal fold on the left. This asymmetry has the advantage of bringing out the configuration of the hypopharyngeal apophyses; on the right side the base of the developing right apophysis is seen, while on the left is seen its more distal end curving round the trito-cerebral ganglion and thereby making connexion with the developing oesophageal dilator muscle. In the premandibular gland a distinction between the two kinds of nuclei has become apparent, but the backward movement of the gland has not yet begun; nor has the duct started to form. × 780.

Fig. 120. 'Horizontal' section through head-end of a 2-day pupa, to show hypopharyngeal apophyses and related structures. Fusion of right and left apophyses has taken place under the oesophagus. In the pre-oral cavity the future suspensorium for the hypopharyngeal apophyses has become defined. The pre-mandibular gland is in process of moving backward into the abdomen. × 780.

Fig. 121. Fragment of a 'horizontal' section along head of a young pupa, to show developing left pre-mandibular gland. The position of the fragment will at once be understood by reference to Fig. 119 or 120. The mandibular apodeme is not present in this section, but the section passes exactly along the epidermal attachment of the gland (indicated by *x*). × 780.

Fig. 122. Similar section, from right side of head of a more advanced pupa, to show pre-mandibular gland in a later phase of development, and with its duct in course of formation. × 780.

Fig. 123. Fragment of a section cut 'horizontally' through head of a young pupa, showing epidermal attachment (*x*) of the maxillary gland. The section grazes the anterior surface of the left maxilla, and passes just under the mandible, which is therefore not present in the section. The rounded clump of cells to the right of the maxillary gland is a fragment of the mandibular apodeme, which intrudes from above into the section. Note also the developing inter-maxillary glands. × 650.

Fig. 124. Approximately similar section, from an advanced pupa; the orientation is not exactly the same, for the maxilla is not present, while the base of the mandible intrudes from above into the section. Note the developing exit duct of the maxillary gland. The maxillary gland has receded into the collum segment. × 650.

Fig. 125, A and B. Two immediately adjacent sections, cut transversely through the hinder-most segments of a young pupa, to show the disposition of the teloblastic mesoderm. The sections are from a 'horizontally' cut pupa (for orientation cf. Text-fig. 9). Section B is 'above', i.e. morphologically posterior to A, and is taken just in front of the anal opening. In section A the last 'ventral organ' is seen on the right side; on the left the section is a little to the rear of it, i.e. it must transect the anal segment itself. In B the section is entirely through the anal segment. In both sections the roof of the fifth abdominal segment is necessarily present in the section. × 780.

Fig. 126. Section through developing sixth abdominal segment of a late pupa; the section is taken at the same level as that shown in Fig. 125 A (right side), the latter section being from an early pupa. Note that the ganglion has begun to enlarge, and that mesoderm cells have spread down the lateral wall of the enlarging ganglion. The small clump of mesoderm cells along the upper margin of the ganglion is the first recognizable sign of the sixth ventral longitudinal muscle. × 780.

Fig. 127. Transverse section through sixth abdominal segment of a very young first instar larva. The drawing is to be compared with Figs. 125 A and 126. Reserve products have not yet appeared in the fat-body. × 780.

Fig. 128. Similar section, but from an advanced first instar larva (cf. Fig. 35). The fifth leg is now developing. Note that the epidermis of the body-wall has grown in thickness, and that along the dorsal surface some of the cells are enlarging, thereby giving the first indication of the development of secondary fat-body. In the old fat-body reserves have appeared in quantity. × 780.

Fig. 129. Section through hinder tip of a young pupa, to show the teloblastic mesoderm. Owing to the fact that the hinder end of the pupa curves upward (cf. Text-fig. 9), the section, whilst transecting the anal segment, must necessarily pass 'horizontally' along the fifth segment. The fifth and teloblastic ganglia, not yet delimited from one another, are therefore cut horizontally, and below this even the hinder tip of the fourth ganglion enters the section. Fragments of the last pair of legs are present. Note also developing fifth ventral longitudinal muscle. × 780.

Fig. 130. Portion of a section through hind end of an advanced pupa. The orientation is about the same as in the previous figure, but development is more advanced. The terminal ganglion has much enlarged, and the fifth abdominal ganglion has partially separated from it. Note, in the anal segment, that the Anlage of the sixth ventral longitudinal muscle has now appeared, behind that of the fifth, having separated away from the clump of teloblastic mesoderm. × 780.

Fig. 131. Transverse section through anal segment of a newly emerged larva, to show teloblastic mesoderm. × 780.

Fig. 132. Similar section, from a later first instar larva. Note pronounced thickening of epidermis, and enlargement of teloblastic mesoderm. Note developing third trichobothrium. × 780.

Fig. 133. Entire Malpighian tube, from a late pupa. × 780.

Fig. 134. The same, from a late first instar larva. × 780.

Fig. 135. The same, from an advanced second instar larva; a fragment of intestine is included at left end of drawing. Note mitosis in one of the cells at base of tube. × 780.

Fig. 136. Transverse section through roof of fifth abdominal segment of an advanced first instar larva, to show an early phase in development of secondary fat-body from the epidermis. × 650.

Fig. 137. Section through roof of one of the hinder segments of a third instar larva, showing development of secondary fat-body from epidermis. The new fat-body is completely closed in above by renovated epidermis. N.B.: this is not a later phase in the development of the part shown in the previous figure, but is from a wholly new part of the larva. × 650.

Fig. 138. Sagittal section of hinder half of a second instar larva. The genital rudiment, with primordial germ-cells located in the sixth abdominal segment, is still in the sexually indifferent condition; the future genital tube is in process of thickening, and is also undergoing terminal elongation at the expense of median mesoderm cells in the growing zone. In the hind-gut note mitosis (indicated by *x*) in one of its epithelial cells. Secondary fat-body is developing, especially along the tergal body-wall. × 385.

PLATE XI

Fig. 139. Portion of a 'horizontal' section of a pupa, to show the genital rudiment. The section passes along the roof of the nerve-cord, and extends from the fifth segment into the hinder end of the third, and therefore includes the genital rudiment for its entire length. Note that behind the primordial germ-cell the genital rudiment merges with the median mesoderm of the growing zone. × 780.

Fig. 140. Section through floor of third abdominal segment of a late first instar larva, showing ingrowing cords of epidermal cells, from which will develop the exit-ducts of the gonads. × 780.

Fig. 141. Similar section from a late second instar larva, showing enlargement of the developing exit-ducts from the gonads. The section is from a larva in which the gonads are already recognizable as male (Fig. 145 is from the same larva). × 650.

Fig. 142. Similar section, from an advanced fourth instar male larva, showing differentiation of the genital ducts. The section is not truly transverse, the left side being a little posterior to the right. Note differentiation of the glandular and anterior portion of ejaculatory ducts. × 650.

Fig. 143. Similar section, from fourth instar female larva, showing degeneration of the left epidermal exit duct, and enlargement of the right duct. Note initial phase in development of receptaculum seminis. × 650.

Fig. 144. Similar section, from a more advanced fourth instar female larva, showing enlargement of the right exit duct (oviduct), formation of receptaculum seminis, and complete disappearance of left exit duct. ×650.

Fig. 145. Transverse section through second instar male larva, taken at the level of the developing hinder pair of testes; cf. Text-fig. 29 A (Fig. 141 is from the same larva). It shows a very early phase in the differentiation of the testes. Note also the distinction between original and secondary fat-body. ×650.

Fig. 146, A, B, C. Three sections through a third instar male larva, to show a more advanced phase in the differentiation of the testes. The gonads of this larva are shown diagrammatically in Text-fig. 29 B.

Fig. A, which represents an almost complete transverse section through the sixth abdominal segment, shows the developing anterior two testes bending dorsally round the mid-gut. In Fig. C, which is taken through the seventh segment, we see the third and fourth testes, only the relevant part of the section having been drawn. In Fig. B, which is a little behind A, and in which again only the relevant fragment has been drawn, we see the narrow strip of genital tube connecting the anterior and posterior pairs of testes. In A and C two cells in meiotic prophase (indicated by x) are present. In B the section passes through the bases of the Malpighian tubes; in C it is well behind them. ×650.

Fig. 147. Section through the upper half of a fourth instar larva, showing later phase in differentiation of testes. The two vasa deferentia are those of the hinder pair of testes. ×650.

Fig. 148. Fragment of a transverse section through the fifth segment of a fourth instar larva, showing splitting of the widened genital tube into the four vesiculae seminales. The section is taken exactly at the point of splitting, which is progressing from behind forwards. ×650.

Fig. 149. Similar section from a more advanced fourth instar larva, showing enlargement of the four vesiculae. ×650.

Fig. 150. One of the hinder testes of an advanced fourth instar larva, cut in sagittal section, and showing spermatogenesis. ×540.

Fig. 151. Transverse section through genital rudiment of a third instar larva, showing the earliest recognizable phase of differentiation of an ovary. Note, on the left, three meiotic prophases. ×780.

Fig. 152. Similar section, from a more advanced third instar larva; prophases of meiosis numerous; follicle cells now present in ovary. ×780.

Fig. 153. Entire ovary and proximal end of genital tube, from a 'horizontally' cut third instar larva. In the ovary we see the first enlarging oocytes. ×780.

Fig. 154. Transverse section through an ovary of a fourth instar larva; enlarging oocytes at the side; germarium in the middle. ×780.

Fig. 155. Parasagittal section along floor of abdomen of a fourth instar female larva, to show condition of the developing reproductive organs. The genital tube is evidently rather asymmetrically placed, for it is contained for its entire length in a section that simultaneously grazes the lateral walls of the chain of nerve-ganglia. On the extreme left is seen the epidermal exit duct, whose connexion with the epidermis, however, lies medial to the section. Then follows the genital tube, with the reproductive cells confined to the sixth and seventh segments. ×385.

The abbreviations used in the Plates are given on pp. 266–7 of Part 1 of this paper (*Quart. J. micr. Sci.*, vol. 88, part 2 (1947)).
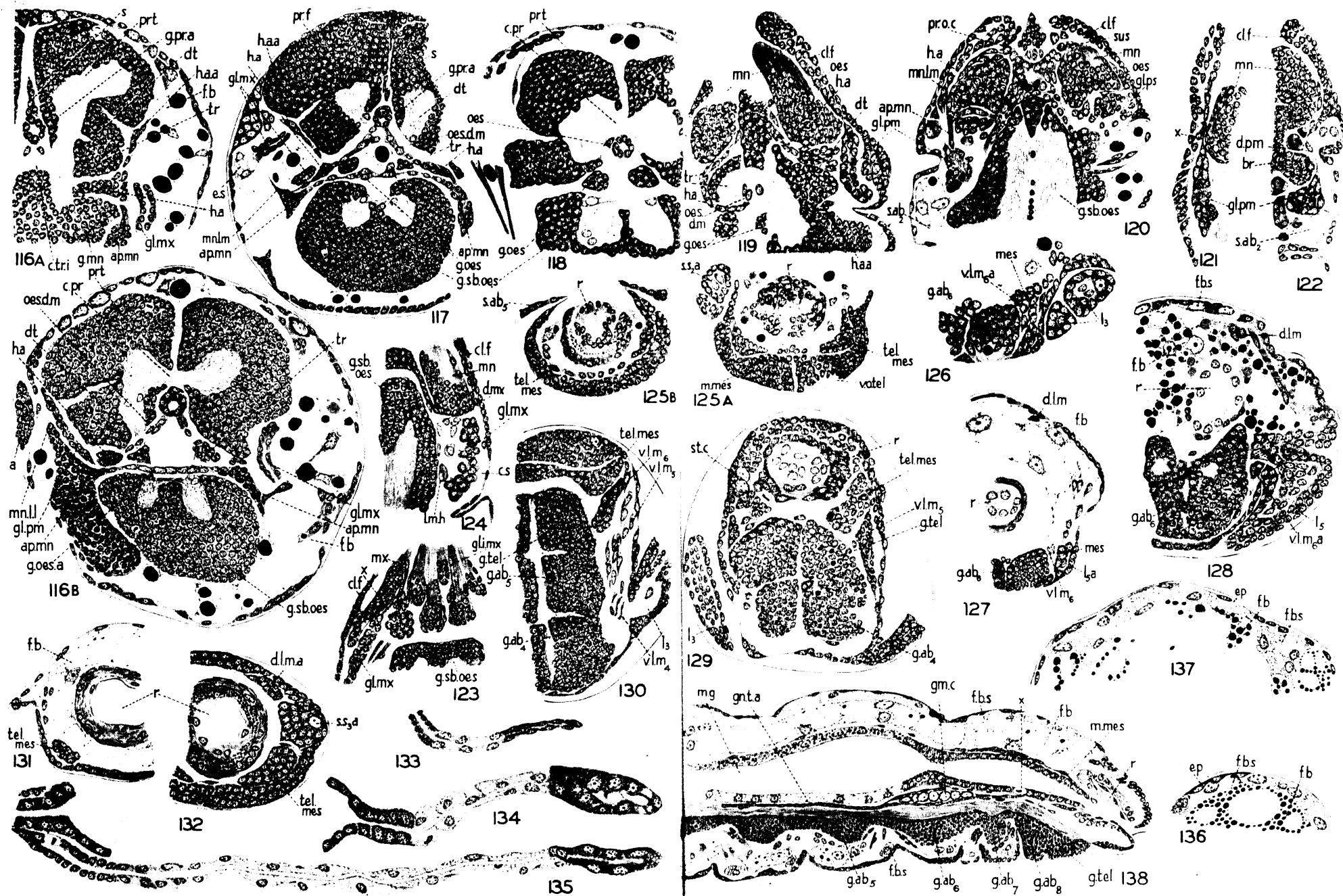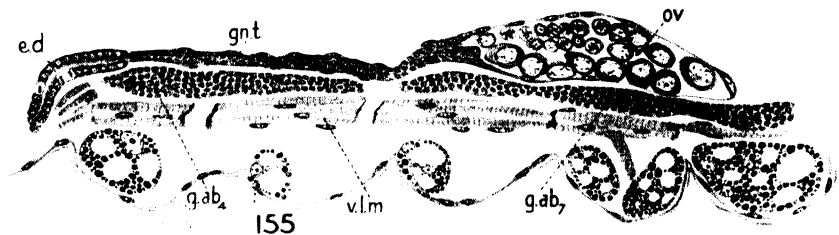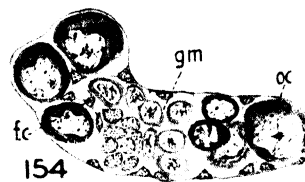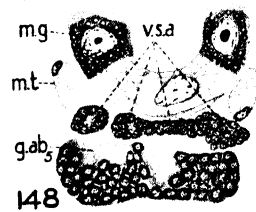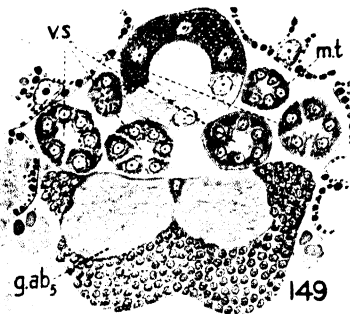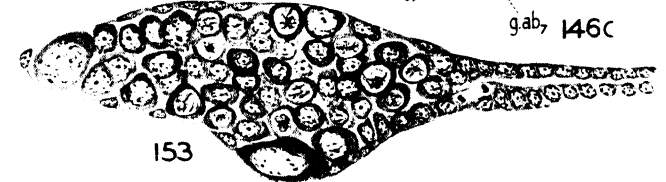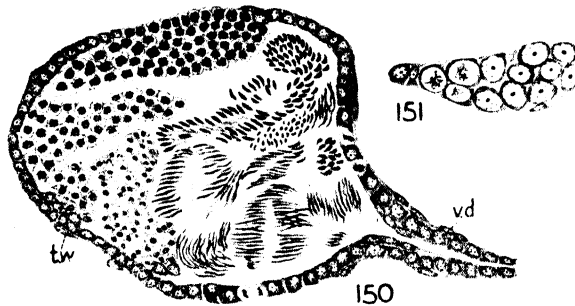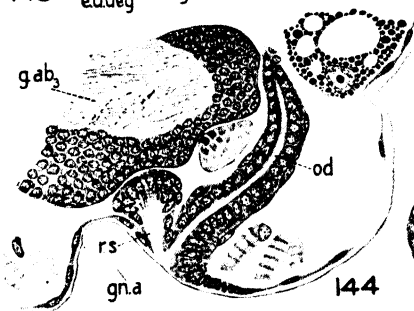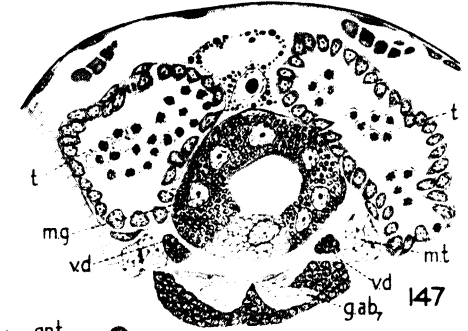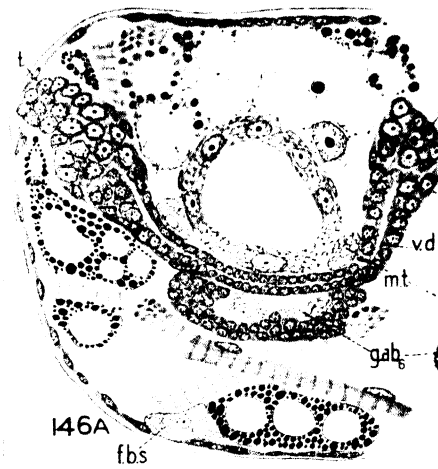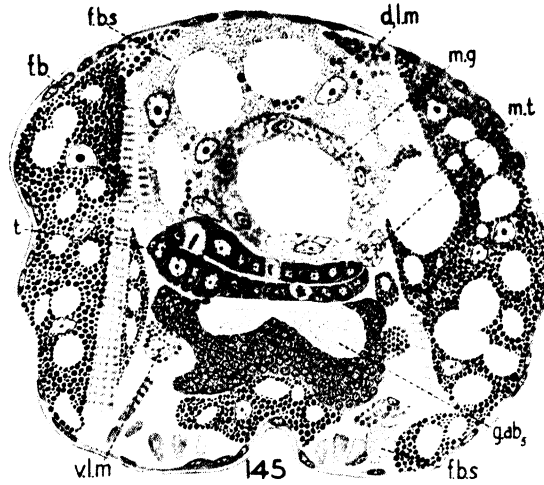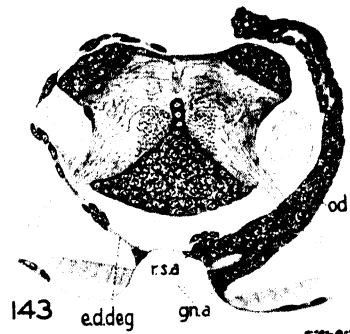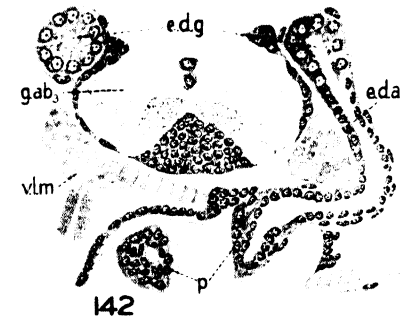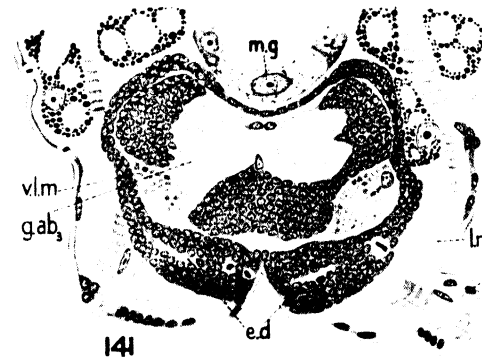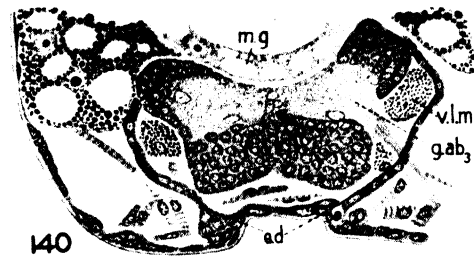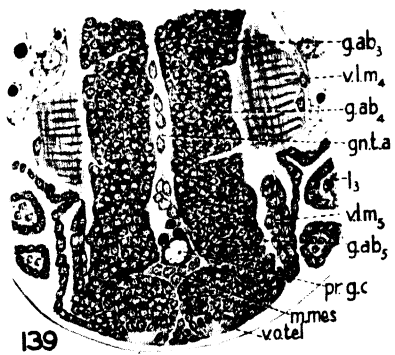
The Phylogeny of the Stomach-Infusorians of Ruminants in the Light of palaeontological and parasitological Data

BY

V. DOGIEL

(*From the Zoological Laboratory of Leningrad University*)

With one Text-figure

IT is a well-established fact that very often the evolution of different groups of parasites progresses on parallel lines with that of their hosts. Only the rate of evolution in the parasites is slower than that of their hosts. While the original hosts of some parasites have time to split into different genera, their parasites hardly diverge so far as to form different species, &c. Very nice cases of such parallel evolution are shown by the lice of man and monkeys, by the nematode *Oesophagostomum* of the same hosts, by some Oestridae of Perissodactyla, &c. In the course of our investigations on a family of endo-parasitic Infusorians, the Ophryoscolecidae, we seem to have met with an especially brilliant case of the same order, which we mean to elucidate in our paper (Dogiel, 1925 et seq.).

Infusorians inhabiting the stomach of Ruminants form one of the most interesting groups of Protozoa. They belong to the family Ophryoscolecidae and dwell in the rumen and reticulum of their hosts. The mode of infection is by direct contact (licking, &c.); cysts are unknown. Ophryoscolecidae were found in all the Ruminants investigated for them: *Camelus*, *Tragulus*, different Cervidae, many kinds of antelopes, *Rupicapra*, goats, sheep, wild and domestic cattle—all of them contain Ophryoscolecidae. The family consists of 10 genera and about a hundred species.

The general morphology of Ophryoscolecidae shows a great uniformity which indicates a monophyletic origin of the family. This assumption is furthermore confirmed by the fact that different genera of Ophryoscolecidae may be very easily arranged in a gradually ascending line of progressive evolution, illustrated by several systems of organelles. In this way the ciliary apparatus, originally consisting of a simple adoral Zone, becomes completed by another, dorsal Zone; the number of contractile vacuoles gradually increases; skeletal plates, primarily wanting, later appear and increase in number and size; the posterior end of the body gradually develops a more and more complex array of caudal spines.

On the first step of this evolutionary ladder has remained the genus *Entodinium*: a simple adoral Zone of membranelles, one contractile vacuole, no skeletal plates, and weakly developed caudal spines are the characteristics of this genus.

The second stage of evolution is occupied by the genus *Anoplodinium*, possessing 2 ciliary Zones, 2 vacuoles, but lacking the skeletal plates; the caudal spines are slightly more developed than in *Entodinium*.

The third grade of morphological evolution is reached by the genus *Eudiplodinium* possessing 2 ciliary Zones, 2 vacuoles, 1-2 narrow skeletal plates, and a scanty caudal armature.

The fourth link of our morphological chain includes the genera *Epidinium*, *Ostracodinium*, and *Opisthotrichum*; they exhibit 2 ciliary Zones, 2-4 vacuoles, 1-3 broad skeletal plates covering the best part of the right side of the body, and well-developed caudal spines.

The fifth and last stage of evolution is represented by *Polyplastron*, *Caloscolex*, and especially by *Ophryoscolex*. These genera possess 2 ciliary Zones, a large number of vacuoles (7--15), often arranged in 1 or 2 transverse rows, a highly developed skeleton consisting of 3-5 plates; the caudal spines reach in the genus *Ophryoscolex* their strongest development, forming from 2 to 4 concentric crowns.

An analysis of distribution of different species and genera of Ophryoscolecidae can give us a good idea of the evolution of this family in those branches of the Ruminants which have been sufficiently well examined by protistologists. These branches are: the family Camelidae (*Camelus*) in the suborder Tylopoda, and families Cervidae and Bovidae (cattle, sheep, goats, and antelopes) in the suborder Pecora. The infusorians of Tragulidae, Antilocapridae, and Giraffidae remain as yet unknown. There exists a small paper by Pringle Jameson (1925) on Ophryoscolecidae of *Tragulus meminna*, but the figures and descriptions are of such a faulty nature as not to be of much use.

Let us now build up a phylogenetic tree of the Ruminants in question, using the well-known treatise of A. S. Romer on *Vertebrate Palaeontology*, so as to see more clearly the points of divergence of different smaller systematic units and the periods when such divergence has taken place. Furthermore, let us apply to the same phylogenetic tree the extant data on the distribution of Ophryoscolecidae in different Ruminants and consider the results. In doing so we must lay especial stress on the species and genera of infusorians strictly limited to certain groups of hosts.

Beginning with the Camelidae we ascertain that the genus *Camelus* possesses 1 unique genus (*Caloscolex*) and 2 unique species (*Entodinium ovum-rajae* and *Anoplodinium cameli*) of Ophryoscolecidae. Other representatives of both the last genera are distributed among all the families and smaller systematic groups of the suborder Pecora, while the genus *Caloscolex*, with its unique species *camelinus*, is restricted to *Camelus* as its only host.

Two deductions seem to follow. The genera *Entodinium* and *Anoplodinium*, which exhibit one or more forms specific to every group of Ruminants inspected, must have arisen before the time when a divergence of Ruminants into Tylopoda and Pecora had taken place, that is, during the Eocene (see Text-fig. 1). It is interesting to note that such an assumption agrees perfectly with the primitive character of both genera (first and second stages of our morpho-

logical ladder of Ophryoscolecidae). On the other hand the genus *Caloscolex* must be of later descent, as we shall presently show.

In the suborder Pecora we encounter two genera, *Eudiplodinium* and *Epidinium*, with numerous species, some of which are specific for Cervidae, other for Bovidae. An obvious inference therefrom is that these genera must

FIG. I
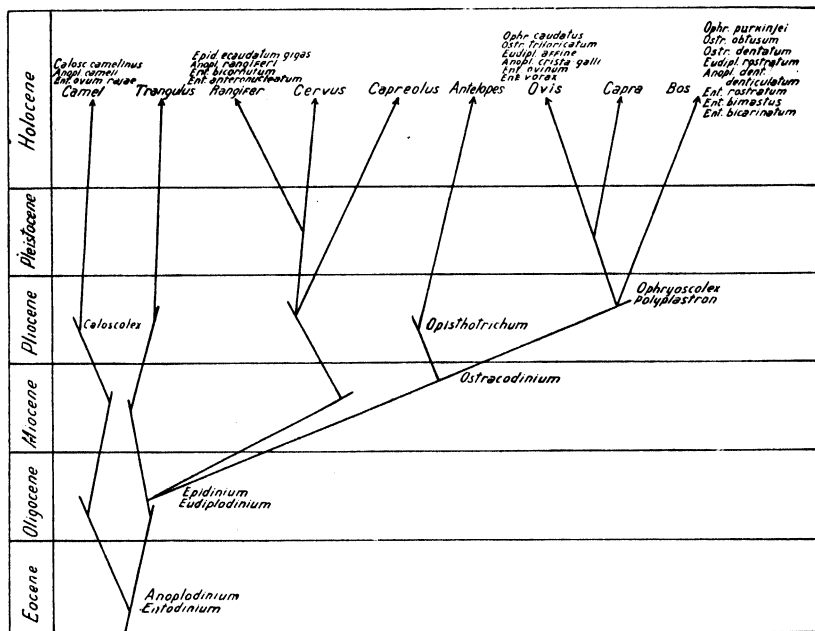
have developed before the divergence of Cervidae and Bovidae was accomplished, that is, in the Oligocene.

The genus *Ostracodinium* is represented by some specific forms in all the subdivisions of Bovidae, but has no species specific for Cervidae. Evidently this genus has arisen after Cervidae and Bovidae became separated, but before the latter branch split into Antilopinae and Bovinae; Miocene must be the time in question.

Following the same line of thought we find that the genus *Opisthotrichum* occurs in several antelopes (*Bubalis cokei*, *B. lichtensteini*, *Madoqua* sp.) only, while the genera *Polyplastron* and *Ophryoscolex* are characteristic of Bovinae (cattle, sheep, and goats). Therefore, the differentiation of these genera has taken place after antelopes and Bovinae became separated, i.e. at the end of the Miocene or the beginning of the Pliocene. A very late appearance of these three genera corresponds exceptionally well with their high grade of organization—they belong to the fourth and fifth stages of our morphological ladder.

The genus *Caloscolex*, found in Camelidae, has reached approximately the same level of morphological evolution as *Ophryoscolex*. That tempts one to make the supposition that *Caloscolex* may have arisen at the same period as its counterpart in the Bovinae. This agrees with the phylogenetic tree of Ruminants, which indicates that the genus *Camelus* (the host of *Caloscolex*) appeared in the Pliocene.

If we follow up the line of the Bovinae we find that the genus *Ophryoscolex* produced species (*O. purkinjei*) to be found only in cattle, while other species are characteristic of sheep and goats (*O. caudatus*). The appearance of these species must be attributed to the end of the Pliocene or even to the Pleistocene (see the phylogenetic scheme).

To the same period may be traced the origin of many other species of different genera which are strictly specific for certain species of Pecora only. Such are, for instance, *Entodinium anteronucleatum* and *Anoplodinium rangiferi* of the reindeer, *Entodinium ovinum, E. vorax, Anoplodinium crista-galli* of sheep and goats, and many different species of infusorians from cattle. All of them appear to have been produced in the Pleistocene.

So far we have been arguing about species strictly host-specific. But there are many species of Ophryoscolecidae inhabiting the stomach of different Ruminantia without any discrimination. Thus, for instance, many common species are to be found in all the domestic Ruminants; some are common to the Bovinae and certain antelopes, while a few may even be encountered in all the groups of Pecora (*Ostracodinium triloricatum*). Such a wide distribution of certain species may be explained in the majority of cases by a secondary exchange of parasitic faunas between different hosts. Such an exchange is especially likely between different species of domestic Ungulates living in close contact. Sometimes certain indirect evidence may point to the real primary host of such species (Dogiel, 1927): *Ostracodinium triloricatum* is primarily a parasite of sheep and goat species, as are also *Entodinium vorax* and *Eudiplodinium affine*, &c. In other cases the primary host remains undiscoverable. Such species we leave undiscussed but they do not vitiate the general course of our idea.

The morphological evolution of Ophryoscolecidae, as traced above, may lead to some conclusions on the physiological evolution of this family. Genera exhibiting a simple general structure are, at the same time, the most ancient ones. These forms (*Entodinium* and *Anoplodinium*) have no skeletal plates and in this respect resemble the great majority of free-living Infusorians. *Entodinium* is more primitive than *Anoplodinium*, being not only of a more simple structure, but also of smaller dimensions. Indeed, the dimensions of Ophryoscolecidae, generally speaking, increase in the course of their evolution.

The food of *Entodinium*, instead of coarse and large grass-particles and splinters of cellulose, consists of spores of fungi, small starch-granules, bacteria, &c. Therefore we may conclude that the ancestors of present Ophryoscolecidae, which for the first time penetrated the stomach of Ruminants, took no part in the assimilation of cellulose in the stomach of their hosts. They were fresh-water Infusorians living close to the shore in shallow parts

of lakes and in water-holes, where they were swallowed by Ruminants drinking at such convenient places. Their food was mixed. They swallowed small detritus-particles, spores, &c., without neglecting other small Protozoa. Such partially predatory habits are suggested by cases of cannibalism regularly recurring in some species of *Entodinium* (*E. vorax*) and *Anoplodinium* (*A. costatum, A. rangiferi*), which feed on smaller species of *Entodinium*. It is especially worth noting that such feeding on smaller Infusorians is to be met with exclusively in *Entodinium* and *Anoplodinium*, that is, in lower Ophryoscolecidae. The higher representatives of the family, although of large bulk and seemingly better adapted for swallowing their smaller relatives, have never been observed to do this. Consequently, predatory habits are to be considered as a survival of the ancient mixed mode of feeding possessed by free-living ancestors of Ophryoscolecidae. Feeding on grass-particles and cellulose was doubtless a later acquirement and signifies a transition to a more specialized mode of feeding.

The last assumption is further suggested by a progressive development of the inner skeleton in higher genera of Ophryoscolecidae. The skeleton in Ophryoscolecidae serves not so much to protect the animal from external injury, as for its support against pressure by large and sharp cellulose-splinters within, especially during the process of engulfing the food. The reason why skeletal plates develop on the right side of the body is precisely to be sought in the right-handed disposition of pharynx, that is, of the channel of introduction for unwieldy food-particles. Long cellulose-fibres, spines, &c. could rend the exterior (right) wall of the gullet, if it was not strengthened by a supporting skeletal plate.

In the higher genera (*Ophryoscolex, Caloscolex, Opisthotrichum*) skeletal plates are most strongly developed and surround the gullet from different sides. The skeleton was developed later and appeared because the Ophryoscolecidae took to a vegetable diet. The chemical composition of skeletal plates (glycogen and hemicellulose, according to Strelkow) also point in this direction. It follows that higher Ophryoscolecidae, in respect of their diet and feeding habits, are strictly specialized forms adapted to their special medium, that is, to conditions in the rumen of their hosts.

In conclusion we may suggest the necessity for complementary studies on Ophryoscolecidae in order to elucidate further essential points in their phylogeny.

1. It is very important to investigate the Infusorians of the lama, huanaco, and vicunha, closely related to *Camelus*, but at present completely isolated from other Camelidae.

2. It is indispensable to reinvestigate the Ophryoscolecidae of Tragulidae. The single work on them made by Pringle Jameson (1925) is not sufficiently reliable. That is the more unfortunate because the Tragulidae are a primitive group of Pecora.

3. Investigation is necessary in several ecologically or systematically isolated species of Ruminants, as in *Antilocapra, Ovibos, Moschus*.

4. But as a most interesting object for investigation we draw attention to the Infusorians of Giraffidae, especially of the okapi. Giraffidae form a separate family among Pecora and may well contain quite a series of new and peculiar Ophryoscolecidae. Furthermore, a study of the Ophryoscolecidae of *Ocapia* could perhaps reveal the provenance of *Troglodytella*, an Ophryoscolecid quite unexpectedly found to infest the gorilla and chimpanzee. We believe that these new hosts may have been acquired by Ophryoscolecidae as a result of infection by the Infusorians of some forest-inhabiting antelopes or okapi. Since *Troglodytella* does not show close relationship to the Ophryoscolecidae of antelopes, we are led to conjecture the possibility of an infection of anthropoid apes *via* the okapi. Infection might occur in the resting-places of anthropoids if they were previously occupied by some Ruminants which left there their saliva or cud.

SUMMARY

1. The members of the family Ophryoscolecidae exhibit several (5) grades of progressive evolution.

2. An analysis of distribution of different species and genera of Ophryoscolecidae indicates the course of evolution of the family in the several branches of Ruminants where these Infusorians have been sufficiently studied; these branches are: family Camelidae and families Cervidae and Bovidae.

3. A comparison of the distribution of different Ophryoscolecidae with the palaeontological occurrence of their hosts leads to the conclusion that the genera of Ophryoscolecidae arose at different periods, the more complicated ones appearing later than the simpler.

4. The genera *Entodinium* and *Anoplodinium* seem to date from the Eocene, while *Eudiplodinium* and *Epidinium* appeared only in the Oligocene; the genus *Ostracodinium* is of a still later descent, and may have arisen in the Miocene. The latest of all to differentiate were the genera *Opisthotrichum, Polyplastron, Ophryoscolex,* and *Caloscolex,* belonging to the fourth and fifth, that is, to the highest grades of morphological complexity. Their origin cannot be traced farther back than the end of the Miocene or the beginning of Pliocene.

5. These conclusions are supported by the differences in feeding habits of different Ophryoscolecidae. The more specialized feeding on cellulose-particles has been developed in the higher Ophryoscolecidae, the lower members of the group (*Entodinium* and *Anoplodinium*) exhibiting a more diversified diet, combined sometimes with predaceous habits.

6. The need for a study of the yet unknown Infusorian fauna of *Lama,* and especially of Giraffidae, is emphasized, and some suggestions as to the origin of an aberrant genus of Ophryoscolecidae (*Troglodytella* from anthropoid apes) are given.

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The Experimental Production of Goblet Cells and Inclusion Bodies

BY

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With 4 Text-figures

IN the course of inoculation experiments on rabbits' corneae we found that at a certain stage large numbers of goblet cells appeared. These are columnar cells with basal nuclei and a varying amount of mucigen in the cytoplasm. The mucigen·distends the cell giving it the shape of a goblet, and finally escapes as mucus from the free border of the cell.

At the same time Ida Mann (1944) drew attention to the occurrence of goblet cells in the cornea during epithelial regeneration in the living eye; these she thought had slid on to the cornea from the conjunctiva. Since this explanation did not seem convincing to us, we decided to follow up this observation by further experiments.

We have been able to demonstrate that goblet cells can arise from normal epithelial cells and that the secretion antecedents originate in the nucleus. The following methods were used to produce these changes:

Method 1. Our first observations were made on corneae subjected to multiple inoculations with human sera. Four weeks after the last injection the cornea showed the following picture (Text-fig. 1). Whilst in the substantia propria no signs of inflammation could be seen except enlargement of the lymph vessels, remarkable changes took place in the epithelial cells. In Text-fig. 1 *a* cells are marked which showed within the nucleus unstainable, glassy, transparent, blister-like bodies, usually looked upon as vacuoles. These increased in size and number till they filled the entire nucleus up to its membrane (Text-fig. 3 *d*). Eventually they were extruded from the nucleus into the cytoplasm, where they again enlarged and multiplied by a process of budding or splitting, giving rise to cytoplasmic inclusions (Text-fig. 1 *b*). By this process the nucleus was pushed to the edge of the cell, became indented, and took on a crescentic shape. A trabecular structure became visible and dots appeared within the strands of it (Text-fig. 1 *c*). These and the whole inclusion stained red with eosin and mucicarmin and sometimes metachromatically with toluidin blue or thionin. There was no longer any doubt that we were dealing with goblet cells.

This observation, unknown in the ontogenesis of rodents, could be reproduced to a considerable degree by the following methods:

Method 2. 0·3 c.cm. of 1,000 e.s.u. Thorium X in sterile saline was injected intracorneally.

Method 3. 350 to 2,000 e.s.u. Thorium X in varnish or ointment was rubbed in after slight scarification of the cornea.

Method 4. Radon 3·4 millic. in 4 c.cm. varnish was used in the same way.

Method 5. A marked response was obtained by rubbing in Thorium X or Radon in the above strengths three times at 4 weeks' interval, followed by injection of filtered human cancer material.
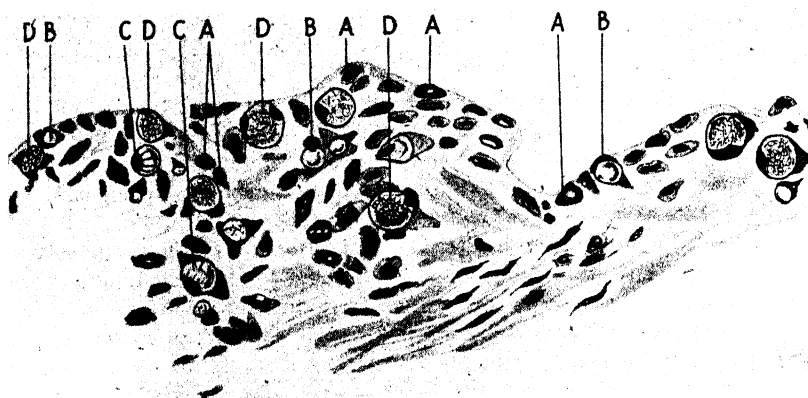
FIG. 1. Nuclear changes in corneal cells. Method 1.

In all these experiments the cornea was injured, and therefore a sliding of the goblet cells from the limbus could not be excluded, but it could be ruled out by the following experiments.

Method 6. The cornea was anaesthetized, cautiously dried, and 2,000 e.s.u. Thorium X applied. After 6 days numerous goblet cells appeared.

Method 7. Radon 3·4 millic. in 3 c.cm. varnish gave the same result.

Method 8. To avoid touching the cornea we exposed it at a distance of 2 inches to short-wave diathermy, electrodes placed parallel and opposite with ⅛ inch spacing, 1 milliamp. for 45 minutes; 25 days later we got the result shown in Text-fig. 2 *d*.

Method 9. 60 minutes' exposure to infra-red rays and

Method 10. 45 minutes' exposure to the Kromayer lamp at 4 inch distance resulted in the formation of goblet cells. Shorter exposures produced only the nuclear inclusions described and precursors of goblet cells.

Localization of the Goblet Cells

As to the areas which are prone to develop goblet cells a rule could not be established. Often they were only found in the first third of the cornea near the limbus, often only in the middle, but by using methods 5–7 every part of
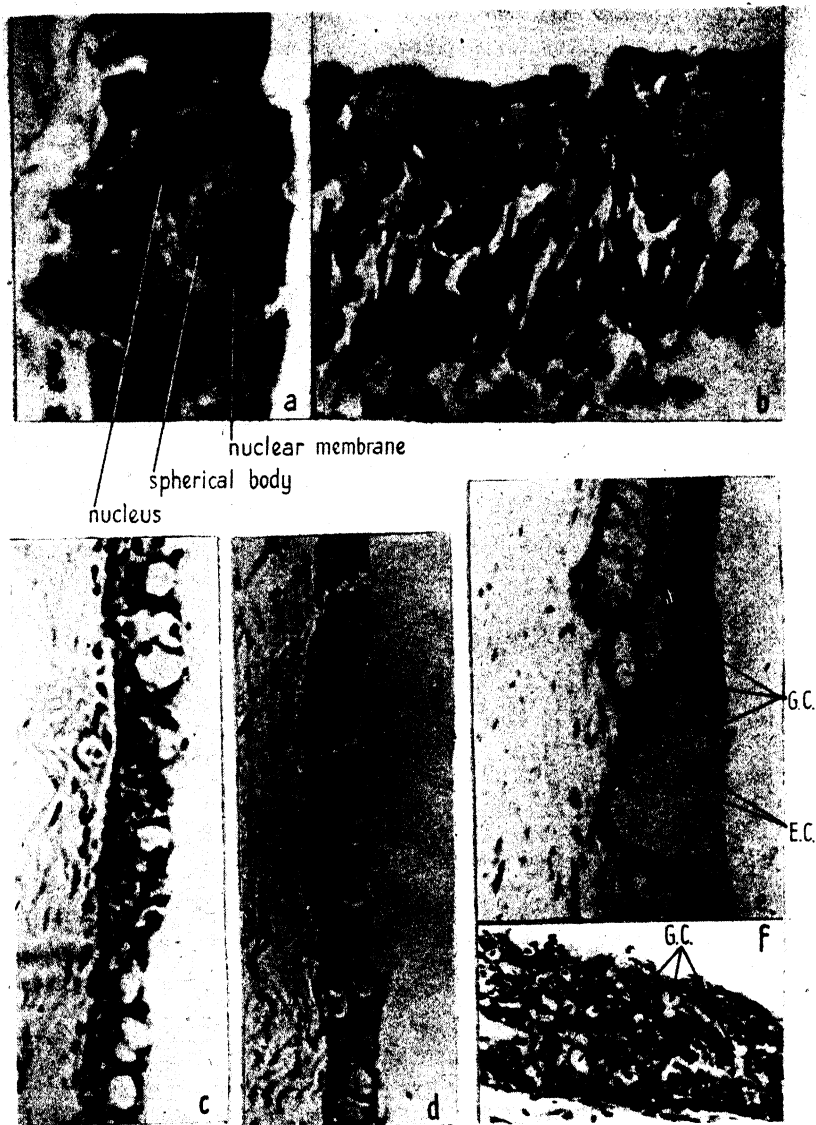
FIG. 2. *a*: Nuclear changes. × 1,200.   *b*: Goblet cells. × 960.   *c, d*: Goblet cells. × 720. *e, f*: Proliferation of goblet cells (G.C.) and epithelial cells (E.C.).   *e*: × 720, *f*: × 400.

the córnea showed goblet cells, sometimes interrupted by areas of normal epithelium (Text-fig. 2 *c*).

Time-table of the Occurrence of Goblet Cells

When using method 6, the precursors of goblet cells could be seen from the second day on, and fully developed goblet cells made their appearance on the sixth day. In methods 1 and 5 we found the greatest number 2 to 4 months after the last application of the stimuli.
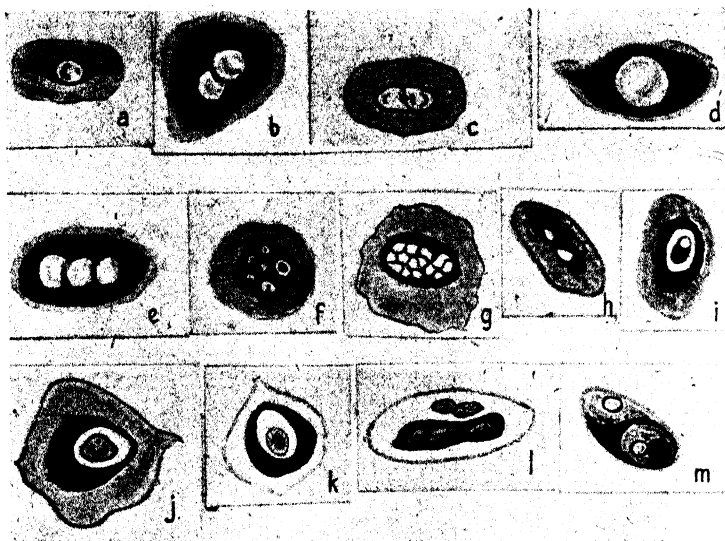
FIG. 3. Nuclear inclusions in corneal epithelial cells after diathermy. *a–h, j, k, l*: haematoxylin-eosin. *i* and *m*: haematoxylin-mucicarmin. × 960.

From the fact that the number of goblet cells increases during the first 4 months and that a proliferation of them into the substantia propria takes place (Text-fig. 2 *e, f*), we concluded that this was an active and not a degenerative process.

One year after treatment the corneae looked normal, except in one case, which still showed a fair number of goblet cells. Assuming that these corneae had reacted in the same way as all the others treated in this way (method 5), we can conclude that in this time the goblet cells disappear again.

Mucous Degeneration for the Surface Epithelium

After application of diathermy, Thorium X, Radon, and occasionally after varnish only, we found a mucous degeneration of the topmost layer (Text-fig. 4 *t, u*). This seemed to have no relation to the appearance of goblet cells.

The Life-history of the Goblet Cells

Having demonstrated that goblet cells can be produced in the normal cornea without sliding from the conjunctiva, and that they develop from ordinary, normal epithelial cells, we could study the striking cytological changes which took place during their development.
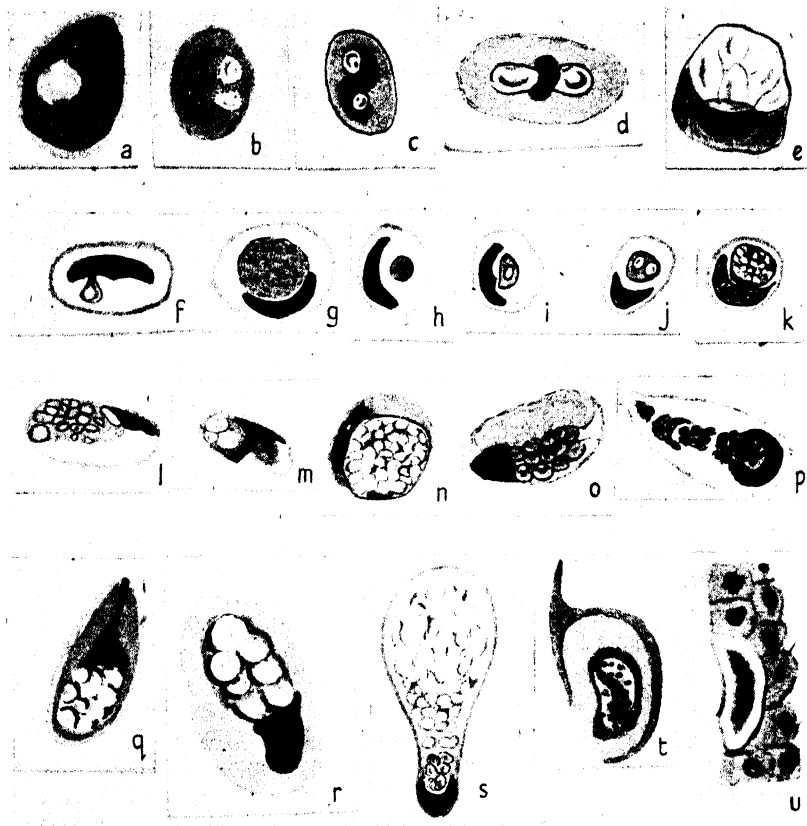
FIG. 4. Cytoplasmic inclusions and goblet cells in corneal epithelium after diathermy. *a–f, q–s*: haematoxylin-eosin. *g–p, t, u*: haematoxylin-mucicarmin. *a–t*: × 960, *u*: × 720.

The first changes observed were in the nucleus. Granules, blisters, or spherical bodies appeared within it (Text-fig. 3 *a*). They increased in size and number by a process of budding or splitting (Text-fig. 3 *b, c, e, f, g*) till they filled the entire nucleus to the point of bursting, leaving only its membrane stained (Text-fig. 3 *d, i, l*). Eventually they were extruded from the nucleus to form cytoplasmic inclusions (Text-figs. 3 *m*; 4 *a–n*).

The following reasons led us to the conclusion that the cytoplasmic

inclusions were derived from the nucleus: (1) Nuclear inclusions were found before cytoplasmic inclusions appeared. (2) When inclusions appear in the cytoplasm they disappear from the nucleus. (3) We often saw inclusions lying partly inside the nucleus and partly in the cytoplasm. In his paper on the effect of ultra-violet light on the cornea Duke-Elder (1929) made the same observation: highly refractile granules appeared within the nucleus and coalesced to form discrete inclusion bodies. They replaced the nucleus and were extruded into the cytoplasm.

The inclusions were at first unstained by the usual histological methods, but at a later stage most of them could be stained by acid aniline dyes, such as eosin, Biebrich scarlet, or light green, and eventually they could be stained with mucicarmin and mucihaematin. Sometimes the cytoplasmic inclusions stained metachromatically with thionin and toluidin blue, which is a specific reaction of sulphuric esters of high molecular weight (Lison, 1936) like mucus. They are Feulgen negative and therefore not composed of thymo-nucleic acid, but they may contain other chromatin substances.

The further development is shown in Text-fig. 4 h–s. After extrusion from the nucleus vacuole-like areas again appear in the inclusions. Again they enlarge and increase in number by budding or splitting and eventually form the mature goblet cell (Text-fig. 4 s), pressing the nucleus to the edge and giving it a crescentic shape.

A Response Mechanism of the Cell to Animate and Inanimate Stimuli

The same nuclear changes were found during our experiments with 362 eyes, irrespective of the stimulus applied. We injected 5–20 per cent. saline, normal and pathological sera, infected the cornea with vaccinia, and exposed it to radiant energy: we always found the nuclear changes described above (Text-figs. 2 a; 3). Similar observations have been reported by Watanobe (1930) in corneas injected with various toxins.

Production of secretory material from within the nucleus has been demonstrated in several other physiological processes. The origin of melanin from nuclear extrusions has been demonstrated by Meirowsky (1906) and since been confirmed by several workers (Meirowsky, 1940). The production of keratohyalin from nuclear and nucleolar material has been observed by Herxheimer and Nathan (1916) and Ludford (1924). Hammar (1897) and Gilmour (1937) described nuclear inclusions in the epithelium lining the male genital tract which Gilmour thought were 'expressions of augmented secretory activity'. Other examples of the secretory function of the nucleus or nucleolus are given by Berg (1935) who describes intranuclear and nucleolar 'blisters' containing fat, pigment, glycogen, and iron in liver cells.

Exactly analogous nuclear changes are found in certain virus diseases, in squamous cell cancer (Meirowsky, 1921), in Paget's disease of the nipple and carcinomatous effusions (Meirowsky and Keys, 1945), and in *Molluscum contagiosum* (Meirowsky, Keys, and Behr, 1946).

These changes have been described in detail by us in another paper (1946).

In earlier literature similar changes have been recorded by Leloir (1878, 'altération cavitaire'), Renaut (1881), Borrell (1903, 'globules blancs'), and Brinckerhoff and Tyzer (1906). In virus diseases they were called 'bodies' by Neisser (1882), 'blisters' by Herzberg (1936), 'extrusion bodies' by Himmelweit (1938), and 'cysts' by Merling (1943).

Suggestions for the significance of these inclusions have been made by Woodruff and Goodpasture (1929), who state that the virus of *Molluscum contagiosum* develops around and later within cytoplasmic vacuoles 'which may be regarded as the cellular response to the presence of a living foreign body'. Evans and Scott (1921) described the 'segregation apparatus' of the cell.

We propose the following hypothesis to explain and co-ordinate our findings: The formation of nuclear inclusions and their extrusion into the cytoplasm is a *constant and essential response mechanism of the cell to stimuli of any kind, animate or inanimate.* If the stimuli are viruses, these are located within the inclusions. If a further development occurs its nature depends on the inherited or intrinsic properties of the cell. In the case of the epithelial cell of the cornea the inclusions may change into mucigen. In other conditions, such as Bowen's disease or squamous cancers, they form hyaline masses, in plasma cells Russel bodies, in *Molluscum contagiosum* they contribute to the keratoid degeneration of the Henderson-Patterson bodies.

The size and number of the inclusions seem to depend on the kind and strength of the stimulus. The changes are small and rare in normal and embryonic tissues, where only very few external or hormonal stimuli are operating. They increase with stronger stimuli, such as tar, but when viruses are attacking the cell or our combined method 5 is employed the inclusions become very conspicuous, and most of all when cancer attacks the cell. It may not be fortuitous that the stimuli which in our experiments produced the largest numbers of inclusions are also carcinogenic.

Controls

Lucas and Herrmann (1935) described the occurrence of cytoplasmic inclusions in the corneae of normal rabbits. We have examined the corneae of 40 normal rabbits and guinea-pigs and we too found occasional cytoplasmic inclusions, comparable in number with those found in normal or embryonic skin. We never found them in such large proportions as did these authors, and it is possible that their animals had been exposed to unknown irritants.

ADDITIONAL OBSERVATIONS

During the course of these investigations certain additional facts were noted. The substantia propria was changed in all cases which were examined at an early date, but no signs of inflammation except enlarged lymph vessels were found when the eyes were examined after several months only. In early cases the substantia propria was infiltrated with all kinds of inflammatory cells. Swelling of the endothelial cells and new formation of blood-vessels was a common feature.

Corneae treated with short-wave diathermy or Thorium X often showed a marked increase in pigment, melanoblasts in the middle of corneae in weakly pigmented animals, and a striking occurrence of pigment in the substantia propria, even when the epithelial cells were not or only slightly pigmented. These observations might have a bearing on the problem of the origin of pigment.

The application of radiant energy usually leads to a cessation of mitoses, amitotic division, and formation of giant cells with 3–6 nuclei in one epithelial cell.

A proliferation of epithelial cells and even of goblet cells into the substantia propria was often observed and is shown in Text-fig. 2, *e* and *f*.

We wish to express our sincere thanks to Dr. R. C. Matson for his kind permission to carry out experiments in the Pathological Department of the Royal Surrey County Hospital, Guildford. By courtesy of the British Research Council we received Radon free of charge. We owe a great debt of gratitude to Mr. P. H. Jacobs for his technical assistance, Mr. D. Moon for his microphotographic work, and to Mr. Bagwell for his help with the animals.

SUMMARY

1. Goblet cells have been produced experimentally in rabbits' and guinea-pigs' corneae by ten methods.

2. Goblet cells can develop from ordinary epithelial cells.

3. The secretion antecedents originate in the nucleus.

4. Intranuclear and cytoplasmic inclusions can be produced experimentally and form the response mechanism of the cell towards animate and inanimate stimuli.

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The Cytoplasmic Components of Germ-cells during Spermatogenesis in the Domestic Fowl

BY

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With 35 Text-figures

Contents

INTRODUCTION

THE investigation here recorded was undertaken in order to determine the changes which the cytoplasmic components, Golgi material and mitochondria, of the male germ-cells of the domestic fowl undergo during the successive stages of spermatogenesis; but nuclear changes during spermateleosis were also very carefully studied.

There is no previously published work on the cytoplasmic components during avian spermatogenesis, so that the present paper gives a full account of these structures, and also contains a detailed description of the metamorphosis of the spermatid. It is worth mentioning that the nuclear changes during spermateleosis were described by some of the older workers and by one recent observer. The results of these investigations will be discussed later in this paper.

MATERIAL AND METHODS

The material used in the present study consisted of the testes of the domestic fowl (*Gallus domesticus*). Very small pieces of testicular tissue were placed immediately after death in fixing fluids, and subsequently sections were cut at $5\,\mu$ and $8\,\mu$ in thickness. Smears were also made.

For the study of the Golgi material, testes were fixed according to the silver-nitrate methods of Aoyama and Da Fano, and the sections were subsequently

stained with Ehrlich's haematoxylin. The osmic method of Kolatchev was also used. It was found that the material treated according to Aoyama's technique gave the best results. Flemming, without acetic acid, and Champy-Kull were used as fixatives for the study of the mitochondria. The sections were stained either with Heidenhain's iron haematoxylin or with acid fuchsin. In this material the acrosome and the centrioles were clearly visible and the Golgi material was stained, but its structure was not shown as clearly as in the silver impregnated material. For the study of the nuclear changes Bouin's fluid proved to be very good. Material was fixed in formol and sections stained in Sudan IV for the demonstration of fat. Drawings were made with the aid of a camera lucida.

The present work was carried out in the Department of Zoology, University of Edinburgh, and it is with the greatest pleasure that I express my thanks to Professor James Ritchie for granting me research facilities, for the interest he has shown in my work, and for reading the manuscript. I should like to thank also very sincerely Dr. R. A. R. Gresson for his useful suggestions and advice.

OBSERVATIONS

The Sertoli Cells. These cells, sometimes called nurse cells, vary in shape and structure. The nucleus is very large, contains a big nucleolus, and is usually stained very lightly. The resting Sertoli cell is found near the connective tissue wall of the tubule; it is elliptical in shape, and at the pole of the nucleus directed towards the lumen of the tubule the Golgi material is localized in the form of a compact mass of argentophil material (Text-fig. 2). The mitochondria are present at the same pole of the nucleus as the Golgi material, they are very numerous, and appear in the form of granules which vary in size (Text-fig. 1). At a certain stage of development of the spermatids the Sertoli cell begins to elongate, and the Golgi material breaks up into granules which extend through the elongate part of the cytoplasm (Text-fig. 3). The mitochondria become scattered in the same way as the Golgi particles. Finally, the elongate part of the Sertoli cell reaches the lumen of the tubule, and groups of late spermatids become imbedded in the cytoplasm of the Sertoli cell. The Golgi particles, previously-scattered over the cytoplasm of the Sertoli cell, now surround each sperm head (Text-fig. 4).

The Spermatogonia. Spermatogonia are usually present at the periphery of the seminiferous tubule. They are elliptical in shape and contain large nuclei; at the anterior pole of the nucleus is situated the Golgi material in the form of a mass of closely packed granules and rods (Text-fig. 5). The mitochondria are spherical and comparatively large in size; they are clumped together in the form of a horseshoe, the centre of which covers the Golgi material, while the two arms embrace the anterior half of the nucleus (Text-fig. 20).

The Spermatocytes. The primary spermatocyte is the largest germ-cell present in the testis; it is situated in the vicinity of the wall of the tubule, next to the spermatogonia. In sections from Flemming and Champy-Kull pre-
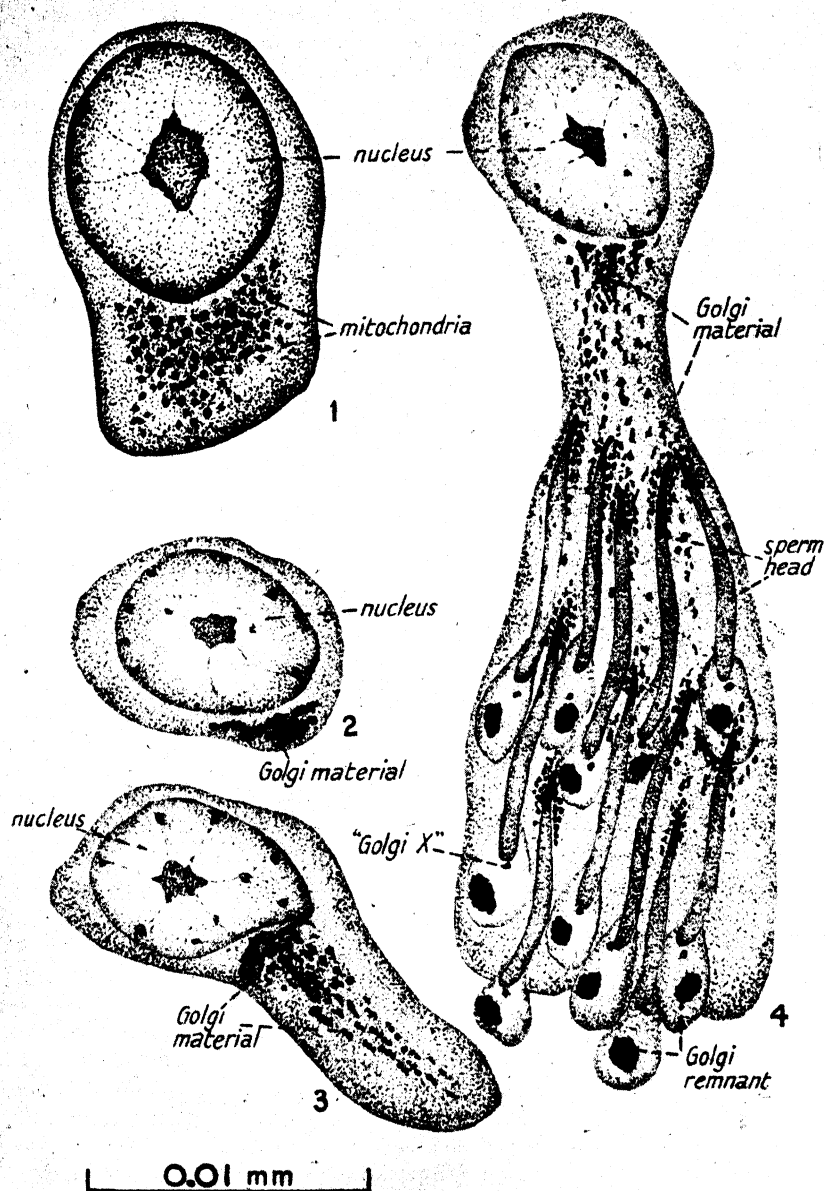
TEXT-FIGS. 1-4

Fig. 1, from Champy-Kull preparations; Figs. 2-4, from Aoyama preparations.

Fig. 1. Resting Sertoli cell to show the mitochondria. Fig. 2. Resting Sertoli cell to show the Golgi material. Fig. 3. Sertoli cell at the beginning of the elongation of the cytoplasm. Fig. 4. Sertoli cell nursing late spermatids.

parations, showing the nucleus in the resting stage, the Golgi material is visible as a lightly coloured homogeneous body in the form of a cap situated at the anterior pole of the nucleus. The mitochondrial layer shows the first signs of dispersal, and the individual granules become unequal in size. In the cytoplasm a darkly stained, small spherical body is often present; this body is called in the present paper the 'accessory body' (Text-fig. 21). At the time when the nucleus enters the prophase of the first maturation division, the Golgi material spreads out along the wall of the nucleus, and assumes a half-moon shape. Inside the Golgi material two small granules of unequal size are visible; these are according to Miller (1938) central bodies. Considering the difference in size between the two bodies, the present writer is rather inclined to think that they are accessory bodies which are formed inside the Golgi material in the same way as in the mammalian spermatocyte. The mitochondria, some of which attain quite a large size, become more or less evenly distributed through the cytoplasm (Text-fig. 22). In sections of silver preparations (Aoyama, Da Fano) the Golgi material shows a very dense cortical layer, which is composed of small rods clumped closely together, and a central layer, brown in colour, consisting of very loosely arranged Golgi rods (Text-fig. 6).

With the beginning of the metaphase of the first spermatocyte division, the Golgi material of the silver preparations breaks up into several clumps which vary in size and become dispersed through the cytoplasm (Text-fig. 7). At the time when the chromosomes are arranged in the equatorial plate a further dispersal of the Golgi material takes place; individual Golgi elements are now scattered all through the cytoplasm with the exception of the area around the asters which remains free of Golgi material. This arrangement of the Golgi elements persists during the subsequent stages (Text-fig. 8). After the division of the cell the dispersed elements of the Golgi material reassemble to form the localized Golgi material of the second spermatocyte. The mitochondria remain scattered during all the division stages, but seem to be absent from inside the spindle (Text-fig. 23). The accessory bodies were not observed during the division of the first spermatocyte.

The secondary spermatocyte is much smaller than the primary, and is only slightly larger than the spermatogonium. The Golgi material in silver preparations is crescentic in shape, with a dense black cortical layer which is present at the convex side of the crescent, and absent from the part next to the nucleus. One or more granules with an argentophil cortex are present in the cytoplasm in the vicinity of the Golgi material. These bodies, called in the present paper 'Golgi X', seem to behave in a similar way to the accessory bodies of the stained chrom-osmium preparations (Text-fig. 9). The Golgi material in the Flemming preparations is crescent-shaped, and its outer edges stain much more deeply than the central part. The mitochondria of the second spermatocyte remain scattered through the cytoplasm. In the cytoplasm one or more, frequently two, darkly stained accessory bodies are present (Text-fig. 24). During the second maturation division the Golgi
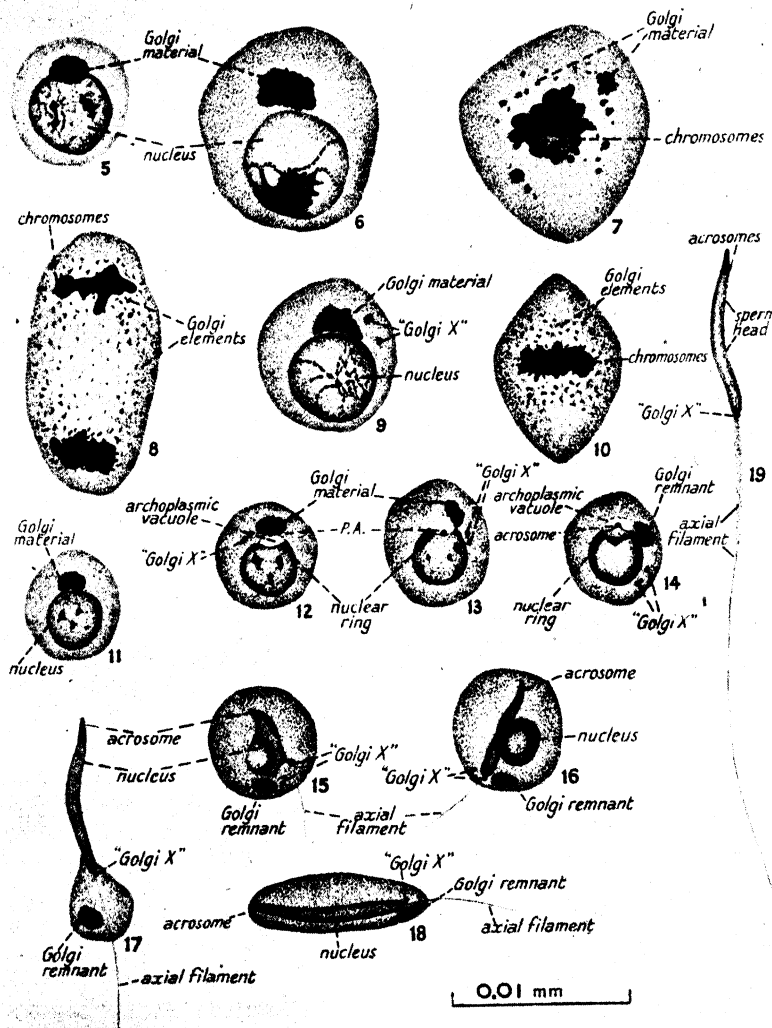
TEXT-FIGS. 5–19. All figures from Aoyama preparations.

Fig. 5. Spermatogonium. Fig. 6. Primary spermatocyte—early prophase. Fig. 7. Primary spermatocyte—early metaphase. Fig. 8. Primary spermatocyte—late anaphase. Fig. 9. Secondary spermatocyte—early prophase. Fig. 10. Secondary spermatocyte—metaphase. Figs. 11–14. Young spermatids. Fig. 15. Spermatid showing the beginning of the elongation of the nucleus. Fig. 16. Spermatid showing a coiled nucleus. Fig. 17. Late spermatid—residual cytoplasm attached to the posterior end of the nucleus. Fig. 18. Late spermatid—straightened-out nucleus inside the cytoplasm. Fig. 19. Spermatozoon.

P.A., proacrosome.

material and the mitochondria behave in a similar manner to those of dividing primary spermatocytes (Text-figs. 10 and 25).

Spermateleosis. The spermatid, which emerges after the second maturation division, is a comparatively small cell with a spherical nucleus; the latter contains, in the early stages, masses of darkly stained material scattered through the nucleoplasm (Text-figs. 11 and 26). With the appearance of the archoplasmic vacuole a depression is formed on the anterior pole of the nucleus (Text-figs. 12 and 28). After the deposition of the proacrosome, the darkly stained masses within the nucleus disappear, leaving the outer edges of the nucleus very deeply stained. With the decrease in size of the archoplasmic vacuole, the part of the nucleus on which the proacrosome is situated begins to grow out in the form of a narrow process (Text-figs. 14, 29, and 30). As this process increases in size, the nucleus assumes a very irregular shape (Text-fig. 30). Very soon the process begins to bend slightly downwards (Text-fig. 15); and later the broad part of the nucleus becomes thinner, and the distinction between the anterior process and the remainder of the nucleus slowly disappears (Text-fig. 31). The bent part of the nucleus grows downwards and reaches the posterior end of the cell (Text-fig. 32). Through continuous elongation of the nucleus, and lack of response from the cytoplasm, the nucleus begins to coil up (Text-fig. 16). Later the cytoplasm begins to elongate; this leads to the uncoiling of the nucleus and to its straightening inside the cytoplasm (Text-figs. 18 and 33). A contraction and shrinkage of the cytoplasm now take place with the resulting outgrowth of the nucleus, which becomes free of cytoplasm along its whole length, apart from the posterior end which still remains in contact with the cytoplasmic remnant (Text-figs. 17 and 34). The next stage in the metamorphosis of the spermatid is the sloughing off of the cytoplasmic remnant, and the nucleus now forms the head of the spermatozoon (Text-figs. 19 and 35). The head of the spermatozoon is narrow, cylindrical, elongate, and worm-like in appearance. It is worth mentioning that the nucleus of the late spermatid is longer and broader than the head of the spermatozoon; this observation would suggest that during the final ripening of the spermatozoon a concentration of the nucleus takes place.

The Golgi material of the spermatid is visible in the stained chrom-osmium preparations (Flemming, Champy-Kull), osmic material (Kolatchev), and in silver impregnated sections (Aoyama, Da Fano). In the Flemming sections the Golgi material appears as a lightly coloured subspherical body situated at one pole of the nucleus. In sections of silver preparations the Golgi material is composed of a number of rods and granules lying on the surface of a brown archoplasm (Text-figs. 11 and 26). Very soon the Golgi material moves away from the nuclear membrane, revealing a clear area—the archoplasmic vacuole which is closely applied to the anterior pole of the nucleus (Text-figs. 12 and 28). After the deposition of the proacrosome, which takes place while the former is situated at the summit of the archoplasmic vacuole, the Golgi material begins to drift down along the edge of the semilunar vacuole and finally reaches the posterior part of the cell. Finally, the Golgi material, now
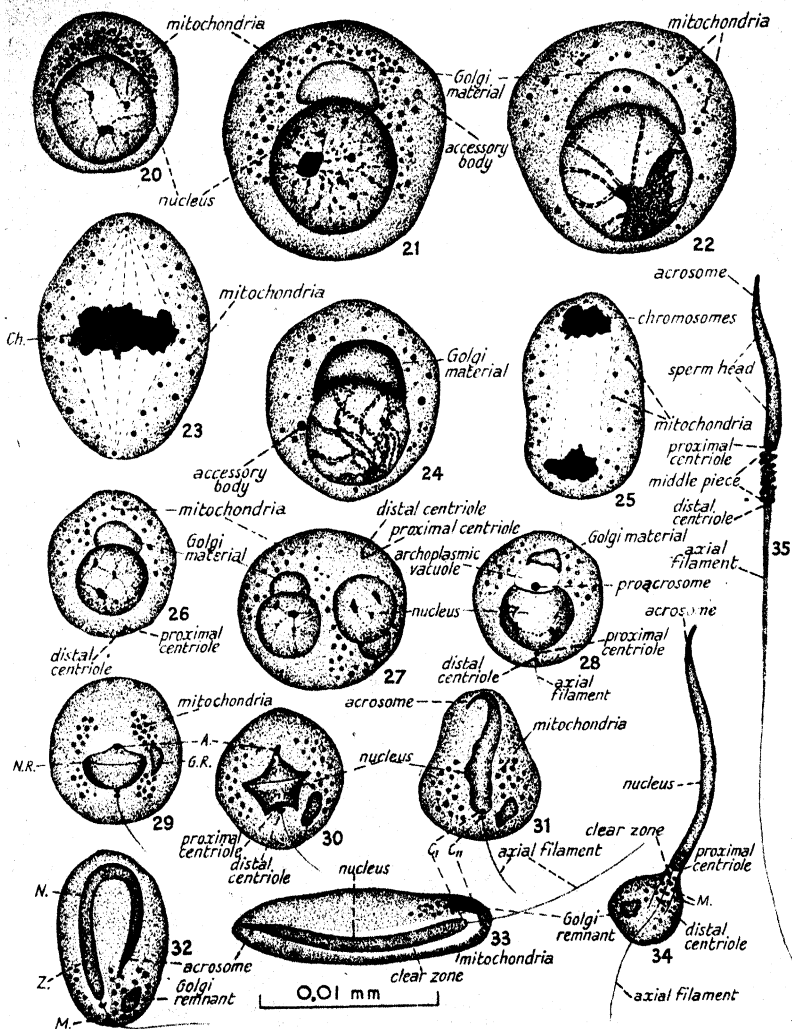
TEXT-FIGS. 20–35. All figures from Champy-Kull preparations:

Fig. 20. Spermatogonium. Fig. 21. Primary spermatocyte—resting stage. Fig. 22. Primary spermatocyte—early prophase. Fig. 23. Primary spermatocyte—metaphase. Fig. 24. Secondary spermatocyte—early prophase. Fig. 25. Secondary spermatocyte—late anaphase. Fig. 26. Young spermatid. Fig. 27. Abnormal spermatid. Figs. 28–30. Young spermatids showing the development of the acrosome and nuclear ring. Fig. 31. Spermatid showing the elongation of the nucleus. Fig. 32. Spermatid showing the beginning of the coiled stage of the nucleus. Fig. 33. Late spermatid—straightened-out nucleus inside the cytoplasm. Fig. 34. Late spermatid—residual cytoplasm attached to the posterior end of the nucleus. Fig. 35. Spermatozoon.

A., acrosome; C_1, proximal centriole; C_{11}, distal centriole; CH., chromosomes; M., mitochondria; G.R., Golgi remnant; N., nucleus; N.R., nuclear ring; Z., clear zone.

called the Golgi remnant, is sloughed off together with the residual cytoplasm (Text-figs. 13–18 and 29–34).

At the time when the archoplasmic vacuole begins to lift up the Golgi material, one or more granules, with an argentophil cortex, separate from the Golgi material. These granules move to the posterior part of the cell much more quickly than the Golgi remnant, and in the majority of sections one granule is seen to be in contact with the posterior end of the nucleus. In the ripe spermatozoon the argentophil granule is situated at the proximal end of the tail in close contact with the posterior end of the head. As these granules are argentophil and were observed to separate from the Golgi material, they are considered to be parts of the Golgi material and are called in the present paper 'Golgi X' (Text-figs. 12–19). These granules were not observed in the stained chrom-osmium preparations.

The history of the acrosome begins with the appearance of the archoplasmic vacuole, which lifts up the Golgi material; at the same time pressure is exerted on the anterior pole of the nucleus with the resulting depression of the nuclear membrane. The edge of the depression, in the form of a circular line, stains much more deeply than the nucleus, and is very similar to the structure observed in mammalian spermatids, where it has been called the nuclear ring (Zlotnik, 1943). At the time when the archoplasmic vacuole reaches its largest size a small dark granule—the proacrosome—is visible close to the Golgi material and near the summit of the vacuole (Text-fig. 12). The proacrosome moves across the archoplasmic vacuole and becomes situated in the centre of the nuclear depression (Text-figs. 13 and 28). Later the part of the nucleus where the proacrosome was deposited begins to grow out; this process is connected with the disappearance of the nuclear depression and a decrease in size of the archoplasmic vacuole. The nuclear ring is at this stage still clearly visible (Text-figs. 14, 29, and 30). Soon the archoplasmic vacuole completely disappears, and the proacrosome, now called the acrosome, increases in size and occupies the anterior tip of the elongate nucleus. Further elongation of the nucleus causes the nuclear ring to become less deeply stained and finally no longer visible (Text-figs. 15 and 31). In the late spermatid and in the spermatozoon the acrosome is conical in shape. It is situated at the anterior end of the nucleus, and in the spermatozoon it takes up one-seventh of the length of the head (Text-figs. 16–19 and 32–5).

In the young spermatid the mitochondria are spherical, and the individual granules are of unequal size; they are scattered evenly in the anterior half of the cell around the Golgi material (Text-fig. 26). With the appearance of the archoplasmic vacuole the mitochondria, although remaining in the anterior half of the cell, appear in sections to be divided into two groups situated on both sides of the vacuole and the Golgi material (Text-fig. 28). After the deposition of the proacrosome and the decrease in size of the archoplasmic vacuole, the mitochondria are arranged in four groups, one on each side of the semilunar vacuole and one on each side of the posterior part of the nucleus (Text-fig. 29). At the time when the acrosomal end of the nucleus

begins to grow out in the form of a thin process, the mitochondria are present on both sides of the nucleus (Text-fig. 30). Later, when the nucleus elongates, the mitochondria are situated on both sides of the posterior part of the nucleus only (Text-figs. 31–3). The mitochondria are present in the anterior part of the residual cytoplasm just behind the nucleus; very soon they become arranged along the axial filament (Text-fig. 34). In the spermatozoon the mitochondria form the mitochondrial sheath of the middle-piece. The middle-piece is a very short structure, about a quarter of the length of the head of the spermatozoon. Owing to the irregular distribution of the mitochondrial granules along the axial filament, the mitochondrial sheath appears very frequently as a spiral structure (Text-fig. 35).

Two small centrioles are present in the spermatid. In the young spermatid, soon after the second maturation division, the centrioles are situated in close contact with each other very near the periphery of the cell (Text-fig. 26). Later, the centrioles move towards the nucleus, and the proximal centriole becomes attached to the nuclear membrane at the opposite pole to the proacrosome, and a thin filament grows from the two centrioles (Text-figs. 28 and 29). With the elongation of the nucleus the proximal centriole becomes embedded into the posterior end of the nucleus, so that it is very often not clearly visible; the distal centriole assumes the shape of a ring and in the subsequent stages of spermateleosis moves away from the proximal centriole, and at the time of the uncoiling of the nucleus it is present at the distal end of the clear zone round the nucleus (Text-figs. 31–3). The migration of the distal centriole continues in the residual cytoplasm, where it marks the posterior limit of the future middle-piece (Text-fig. 34). In the ripe sper-matozoon the proximal centriole is attached to the posterior end of the head, and is only visible when the tail is broken off; the distal centriole only is observed at the lower end of the middle-piece (Text-fig. 35).

At the time of the uncoiling of the nucleus a clear zone appears around the posterior half of the nucleus. This zone can be compared with the manchette of mammalian spermatids (Text-figs. 32 and 33). The similarity to the manchette is especially striking in the residual cytoplasm; here the zone is cylindrical in shape, the upper end being attached to the nucleus, while the posterior end remains open. The length and width of the zone in the residual cytoplasm and the arrangement of the mitochondria inside the zone along the axial filament would suggest that the zone marks the boundaries of the middle-piece of the spermatozoon (Text-figs. 34 and 35).

Several abnormal spermatids were observed; these were large cells with either two or four nuclei, each nucleus having its Golgi material and mito-chondria. This is probably caused by a failure of the cytoplasm to divide after nuclear division of the spermatocytes (Text-fig. 27).

DISCUSSION

As already pointed out no previous detailed work has been recorded on the structure and behaviour of the Golgi material during spermatogenesis in birds.

The results of the present investigation show that there is no substantial difference between the structure and behaviour of the Golgi material of avian and mammalian male germ-cells. The Golgi material in the spermatocytes of the domestic fowl consists of a number of rods and granules which surround the archoplasm. During the maturation divisions the Golgi material is scattered through the cytoplasm, and finally the individual Golgi elements become sorted out between the two daughter cells. After the formation of the acrosome, the Golgi material of the spermatid migrates to the posterior part of the cell. The Golgi material, now called the Golgi remnant, does not take any further active part in spermateleosis, but remains in the residual cytoplasm and is eliminated together with the latter.

Several writers record the presence of accessory and chromatoid bodies within the cytoplasm of mammalian male germ-cells. Gresson and Zlotnik (1945) give a full account of the literature on the subject, and they suggest that chromatoid and accessory bodies are identical structures. According to their work, accessory bodies are formed inside the Golgi material of spermatocytes and spermatids and later passed into the cytoplasm, where they remain during the subsequent stages of spermatogenesis. At least one such body is included in the neck region of the spermatozoon. Bodies corresponding to those observed in mammalian germ-cells are present also in the domestic fowl. In the stained chrom-osmium preparations they are visible only in the spermatocytes, while in the silver impregnated material they are present in the spermatocytes and also in the spermatids. One or more, usually two, such bodies occur in the spermatids. In the ripe spermatozoon only one argentophil granule is present in the neck region. As these bodies have an argentophil cortex, and were observed to separate from the Golgi material, they were called in the present paper 'Golgi X'.

The origin of the acrosome in the male germ-cells of the domestic fowl is on the same lines as that described for mammalian germ-cells by Papanicolaou and Stockard (1918) for the guinea-pig, Gatenby and Woodger (1921) for the guinea-pig, Gatenby and Beams (1935) for man, Gresson (1942) for the mouse, and Gresson and Zlotnik (1945) for sheep, pig, dog, cat, rat, rabbit, and golden hamster. As far as avian spermatogenesis is concerned, Brunn (1884) claimed that in the domestic fowl the acrosome is formed in the same way as in mammalian germ-cells and is protoplasmic in origin; in the pigeon, however, the acrosome is of nuclear origin. In the spermatid of the pigeon, according to Brunn, the nucleus divides into two hemispheres, an anterior lightly stained part which contains a nucleolus, and a posterior darkly stained portion. The anterior part deposits a small granule on the prominent tip of the posterior nucleus; this granule is the acrosome. Brunn concludes that the posterior nucleus forms the head of the spermatozoon, while the anterior part of the nucleus serves as a cap for the head. In my opinion the anterior part of the nucleus observed by Brunn is the crescentic Golgi material which is raised up from the nucleus and deposits the proacrosome. Benda (1903) considers the anterior part of the head of the spermatozoon of the

pigeon to be the perforatorium which, according to him, originates from archoplasmic material. Miller (1938) states that the acrosome of the domestic fowl is formed from idiosome material. He figures the acrosome as a group of fine granules situated at the tip of the elongate nucleus.

The mitochondria of the domestic fowl conform more or less to the same rules of behaviour as in mammalian male germ-cells. In the spermatogonia they form a horseshoe-shaped structure around the Golgi material; in the spermatocytes they scatter, and in the spermatids they are present, at first, in the anterior part of the cell, but later they move to the posterior region where they surround the anterior part of the axial filament. In the spermatozoon the mitochondria form the mitochondrial sheath of the middle-piece. Owing to certain irregularities in the arrangement of the mitochondria along the axial filament, the sheath looks sometimes like a spiral structure. Ballowitz (1888) considers the part of the sperm-tail of the pigeon surrounded by the spiral structure to be the middle-piece. Benda (1903) states that the spiral structure, present in the spermatozoon of the pigeon, is composed of mitochondria, and that it surrounds not only the middle-piece, but a large part of the head also. Retzius (1909) disagrees with Benda, and supports Ballowitz's statement; according to him a spiral sheath surrounds the anterior part of the axial filament only.

As to the nuclear transformations during spermateleosis, which are fully described in the present paper, there exists some diversity of opinion amongst the previous workers on avian spermatogenesis. Brunn (1884) believes that the head of the spermatozoon of the pigeon develops through spiral entangling of the elongate nucleus. Guyer (1909), who claims the presence of 8 bivalent chromosomes and 1 accessory chromosome in the primary spermatocyte of the domestic fowl, states that the transformation of the nucleus of the spermatid comes about through the arrangement of 4 or 5 chromosomes in a more or less closed ring, around which the nuclear membrane is formed. The chromosomes concentrate gradually towards one side in the form of a crescent. The nuclear membrane thins out along the margin free of chromosomes and finally fades away, leaving a dense elongate chromatin mass. This mass grows still further and becomes the head of the spermatozoon. In the pigeon Guyer observed a spiral twisting and uncoiling of the nucleus. According to Miller (1938), the first nuclear change during spermateleosis is a spinning out of the chromatin spheres into a network, and an accumulation of chromatin along the nuclear membrane. The network of chromatin material later begins to contract laterally. It is certain from Miller's description that he did not observe the coiled stages of the transformation of the nucleus.

In the present paper two centrioles are described; they are visible in the early spermatid and in the subsequent stages of spermateleosis. The proximal centriole becomes attached to the posterior end of the elongate nucleus, while the distal centriole assumes the shape of a ring, migrates down along the axial filament, and marks the posterior limit of the middle-piece. The axial filament is described as a filament which grows out from both centrioles. Similar

observations were made by Retzius (1909), who states that in some cases at the posterior end of the middle-piece of the spermatozoon of the domestic fowl a ring-shaped structure is present; this body represents the distal centriole. The proximal centriole is attached to the head of the spermatozoon at its junction with the tail, and is clearly visible after the tail is broken off. The results of Guyer's (1909) work agree with those of Retzius. According to Guyer the axial filament emerges from the nucleus of the spermatid in the domestic fowl; at the point where the filament leaves the cytoplasm a ring-shaped centriole is present. Guyer makes it clear that the axial filament, although emerging from the nucleus, is not of nuclear origin, but originates from the proximal centriole which is embedded inside the nuclear substance. The proximal centriole is a very small granule, and in successful preparations is seen to consist of three parts. The view that the proximal centriole divides into three granules is also expressed by Retzius. Miller (1938) states that he observed only a few details of the formation of the tail. In the resting spermatid a central body is present inside the idiosome. Later, the central body elongates into a rod; the latter is at first situated tangentially to the nucleus, but soon turns and moves towards the nucleus until one end touches the nuclear membrane and penetrates the nucleus. The rod-like centriole increases in length, and its posterior end passes out of the cell. As the rod elongates it becomes thinner and stains less intensely. After the elongation of the cell a dark staining material whose origin could not be determined is present at the point where the axial filament crosses the posterior end of the clear zone around the nucleus. The present writer does not agree with Miller that in the resting spermatid the centrioles are situated within the idiosome, as according to the present work the centrioles are set free in the cytoplasm after the second maturation division. Further, in my opinion, the rod-shaped central body described by Miller is in reality the proximal and distal centrioles situated very closely together, and that the darkly stained material present along the axial filament is the distal or ring centriole imperfectly preserved.

Miller (1938) states that after the elongation of the nucleus the latter is seen lying in a clear space surrounded by a membrane; later this membrane flattens against the sperm head and tail. A similar structure was observed in the present investigation and was called for convenience the clear zone. A suggestion is made that this zone plays the same part in avian spermatogenesis as the manchette in mammalian male germ-cells. The similarity to the manchette is especially striking in the residual cytoplasm.

SUMMARY

1. The Golgi material of the male germ-cells of the domestic fowl is in the form of a localized body, composed of rods and granules which lie on the surface of the archoplasm.

2. During the maturation divisions the localized Golgi material breaks down and forms a number of granules and rods which become dispersed at the beginning of the metaphase. After each division the scattered Golgi

elements reassemble in the daughter cells and become once more attached to the archoplasm.

3. After the formation of the acrosome the Golgi material migrates to the residual cytoplasm and, as the Golgi remnant, is eliminated.

4. Accessory bodies are present in the cytoplasm of the spermatocytes from both silver and chrom-osmium preparations; in the spermatids only argentophil bodies are identified. One argentophil accessory body 'Golgi X' is included in the neck region of the spermatozoon.

5. The acrosome is formed from the proacrosome; the latter originates within the archoplasmic vacuole inside the Golgi material of the spermatid. The acrosome occupies the anterior tip of the head of the spermatozoon, and is conical in shape.

6. The nuclear ring is present in the early stages of the metamorphosis of the spermatid; with the elongation of the nucleus it disappears.

7. The mitochondria are described during all the stages of spermatogenesis; their ultimate fate is the formation of the mitochondrial sheath of the middle-piece. This sheath often appears in the form of a spiral structure.

8. During the transformation of the nucleus a coiled stage is constant; this is due to the continuous elongation of the nucleus and lack of response from the cytoplasm. The head of the spermatozoon is an elongate worm-like structure.

9. Two centrioles are present in the spermatids of the domestic fowl. The axial filament originates from the two centrioles. The proximal centriole is attached to the posterior end of the head of the spermatozoon, while the distal centriole assumes the shape of a ring and marks the distal limit of the middle-piece.

10. A clear zone surrounds the posterior part of the nucleus of the late spermatid; this zone possibly takes part in the formation of the middle-piece.

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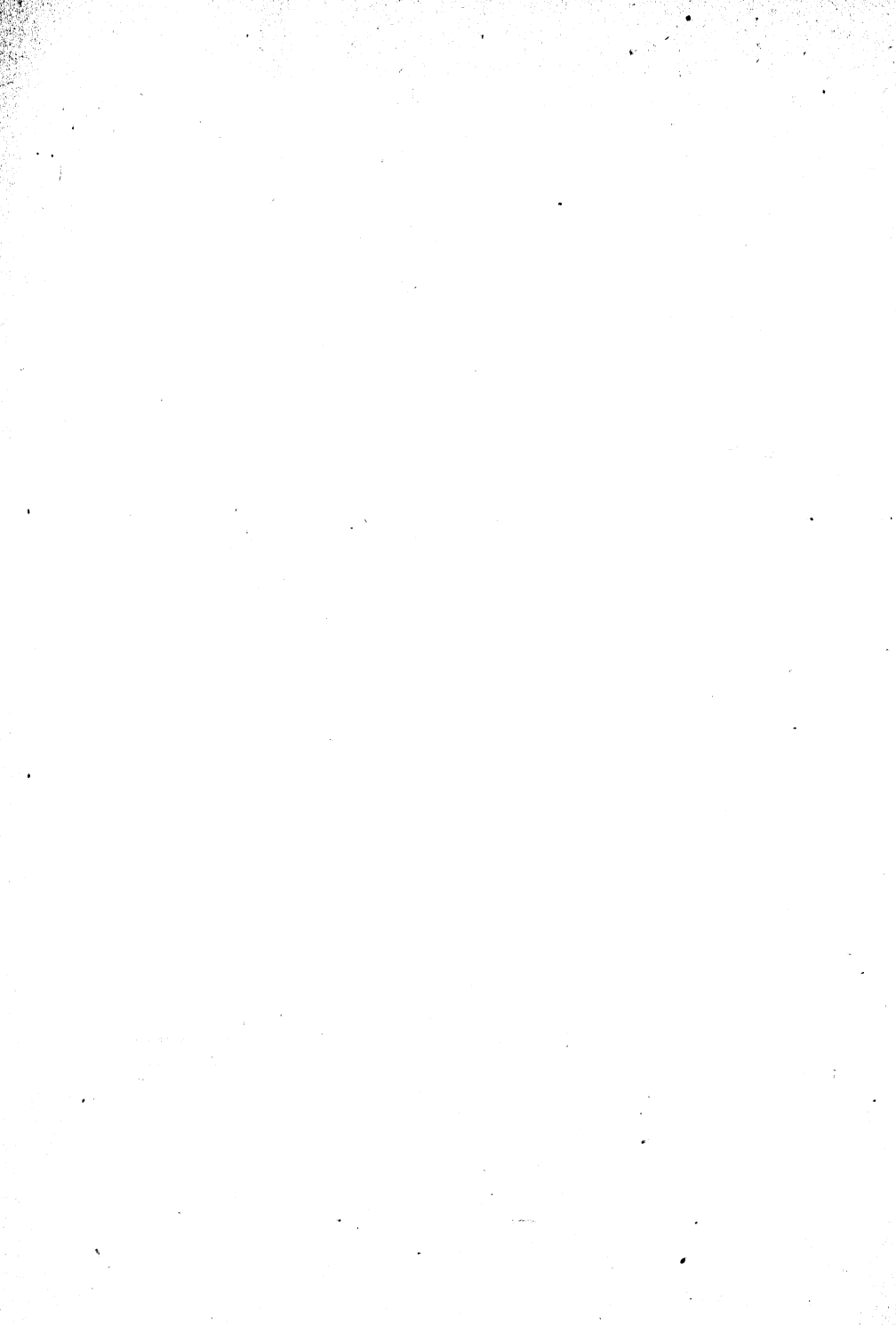
Localization of Alkaline Phosphatase in Mammalian Bones

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With ten Text-figures and one Plate

INTRODUCTION

ALTHOUGH it is well established that alkaline phosphatase is present in high concentrations in mammalian bones, the histological and cytological distribution of the enzyme in normal adult bones has not been adequately studied. Since the introduction of techniques for the histochemical localization of phosphatase (Gomori, 1939; Takamatsu, 1939) a number of papers have been published on the distribution of the enzyme in the developing bones and teeth of mammalian embryos (Gomori, 1943; Horowitz, 1942; Bevelander and Johnson, 1945; Engel and Furuta, 1942) and in the skeleton of fowl embryos (Moog, 1944). The relation of phosphatase to ectopic bone in bladder transplants (Gomori, 1943) and phosphatase in healing experimental fractures (Bourne, 1943) has also been studied.

The reasons why the original Gomori technique was only reluctantly applied to adult bones are twofold:

1. The decalcifying fluids commonly used in histology destroy alkaline phosphatase.

2. Even when reasonably thin sections of undecalcified bone had been obtained there remained the problem of distinguishing between the preformed phosphate and the phosphate deposited during incubation with the substrate.

The first obstacle was partially overcome by Kabat and Furth (1941) who used diammonium citrate to decalcify bones of human foetuses. Bourne (1943) circumvented the decalcifying problem by incubating thin bone slices and, *after* visualizing the phosphatase, decalcified with trichloracetic acid and embedded in the usual way. This method, although it may be suitable for indicating the presence of phosphatase in a roughly qualitative way, is quite unsuitable where the exact cytological localization of the enzyme is to be ascertained, or a quantitative comparison to be made. The reason for this is that the reaction products obtained during the process of converting the calcium phosphate to cobalt sulphide are all more or less soluble and there may be considerable loss and displacement of precipitate during the comparatively long times required for each reagent to penetrate a bone slice. Even with

paraffin sections these factors must be borne in mind if reliable results are to be obtained (Danielli, 1946).

The second difficulty could be overcome in three ways:

(a) By a careful comparison of the incubated bone section with a control section treated identically except for the incubation with glycerophosphate. This is the method usually adopted. Apart from being tedious it does not solve the problem of the localization of phosphatase in an area also containing a high concentration of preformed phosphate, e.g. in the bone matrix. According to Bourne (1943), only the newly formed matrix stains positively, the older bone being negative. I was unable to confirm this.

(b) By adopting a two-colour scheme for preformed phosphate and phosphate liberated through enzyme activity. This is the principle underlying Gomori's (1943) method for bone. The existing calcium salts are converted to the black cobalt sulphide by treatment with cobalt nitrate followed by ammonium sulphide. The section is then incubated with glycerophosphate in the usual manner and the phosphate liberated visualized by conversion to lead phosphate which is stained purple with acridine red. By this method the phosphatase sites can be located fairly well in thin undecalcified sections provided the concentration of preformed phosphate is not so great as to mask the purple of the phosphatase reaction.

The following objections may be raised against the method:

1. Regarding the visualization of the preformed phosphate, it must be borne in mind that ammonium sulphide is an inhibitor of phosphatase (Fell and Danielli, 1943); thus the necessity of adding this reagent to the substrate during incubation, in order to depress the solubility of the cobalt sulphide deposited in the sections, is a disadvantage where low concentrations of phosphatase are to be demonstrated.

2. The lead nitrate–acridine red method of visualizing phosphatase is unsatisfactory with the brands of acridine red available in this country. Using acridine red (Gurr), I found that exact localization of phosphatase was impossible since the coloured lake is easily soluble in the reagents used during dehydration and clearing of the slide and a diffuse picture is obtained. Moreover, acridine red acts as a nuclear stain, thus making the detection of nuclear phosphatase impossible.

If it is desired to show the location of bone salts as well as phosphatase, the following modification of Gomori's (1943) method was found useful: preformed phosphate is shown by conversion to cobalt sulphide and the section incubated in the presence of one drop $(NH_4)_2S$ per 100 c.cm. substrate. After incubation the slide is washed in dilute $Ca(NO_3)_2$ and placed for 10 min. in a saturated buffered solution of gallamine blue (Gurr) at pH 7·0. The slide is rinsed quickly (15–30 sec.) in 0·5 NaOH, washed, dehydrated, cleared, and mounted.

Gallamine blue forms a purple lake with calcium and is specific for this metal (Stock, personal communication); thus the sites of phosphatase activity

appear purple. In neutral solution there is only a slight degree of background staining which is completely removed by the treatment with dilute NaOH. Since the dye-lake tends to redissolve both in the staining bath and in the NaOH, the above times must be strictly adhered to.

The sections may be counterstained with eosin, safranin, or, if it is desired to stain nuclei, methyl green or neutral red. This method is only suitable for tissues in which the initial phosphatase content is high, such as sections of developing mammalian bones.

(c) The difficulty of demonstrating phosphatase in the presence of bone salts may be overcome by using a phosphate ester as substrate and utilizing the organic radicle liberated by the phosphatase to visualize sites of enzyme activity by formation of an azo-dye. This method was first described by Menten, Junge, & Green (1944), who employed Ca β-naphthyl phosphate as substrate; β-naphthol is liberated which, in the presence of diazotized α-naphthylamine, forms a coloured compound. Various modifications of this technique are described by Danielli (1946). He, like Menten, Junge, and Green, applied the method to kidney sections. There is no record of its application to bone sections. Yet a method involving the deposition of an azo-dye instead of visualizing inorganic phosphate should on principle be ideal for calcified tissues since the question of differentiation between pre-formed and newly deposited phosphate does not arise. Unfortunately the method of Menten, Junge, and Green is much inferior to the Gomori–Takamatsu method from the technical point of view. 'Incubation' has to be carried out below 10° C. to avoid disintegration of the diazotized amine. Even so the diazonium hydroxide is destroyed in 2 hours or less. This means constant renewal of substrate as the incubation periods required are considerably longer than for the Gomori–Takamatsu method. The reason for this is not only the low temperature but the fact that phosphatase does not act as readily on the Ca β-naphthyl phosphate in presence of diazotized amine as it does on the glycerophosphate in the Gomori–Takamatsu substrate. Menten, Junge, and Green's method is somewhat improved by the use of α- instead of β-naphthyl phosphate, but even so its sensitivity is low (Lorch, 1947). When applied to undecalcified sections of costo-chondral junctions only the areas of very intense phosphatase activity are picked out.

It is seen that a decalcifying method leaving the phosphatase intact enables one to obtain thin sections of bones irrespective of their initial hardness and, provided decalcification is complete, the original Gomori–Takamatsu method can then be used to visualize the phosphatase. The necessity for unincubated controls or double coloration is thereby eliminated.

MATERIAL

The bones described in this paper were taken from adult rats and mice and from two kittens, one shortly before birth and one newly born. Sections were cut through the ribs so as to include the costo-chondral junction. Long bones are represented by the head of the humerus and the distal end of the femur,

which were cut longitudinally so as to include the epiphyses. The frontal served as an example of membrane bone.

<div align="center">METHODS</div>

A preliminary account of the decalcification method was given in *Nature* (Lorch, 1946). Minor modifications have been made. Cloetens (1942) has shown that alkaline phosphatase extracted from mammalian kidneys can be reversibly inactivated by incubation with acid media. He found that various metallic ions, notably $Zn^{\cdot\cdot}(10^{-4}$ M) had a retarding effect on the course of inactivation, i.e. protected the enzyme. In a second paper Cloetens (1944) showed that the reactivation of the enzyme at an alkaline pH can be facilitated by certain organic compounds, especially by glycine. It was thought that the above facts could be utilized in a method for decalcifying bones with preservation of phosphatase.

The effect of different buffers between pH 4 and 6 on alkaline phosphatase in kidney sections was tested, and Cloeten's findings regarding the protective action of $Zn^{\cdot\cdot}$ ions and the reactivating effect of alkaline solutions containing glycine were confirmed. The buffers were then applied to rat ribs to test their decalcifying power. Optimum conditions for decalcification and reactivation were ascertained. Of the fluids tested magnesium citrate (as recommended by Shipley, 1937) and sodium acetate (Peters and Van Slyke, 1932) were found to be very slow decalcifying agents, but the destruction of phosphatase was very small in the presence of $ZnSO_4$ and with suitable reactivation. The buffer finally adopted as most suitable in every respect was sodium citrate-HCl at pH 4·4–4·6 (Clark, 1928).

The following procedure is recommended:

1. Small pieces of bone are fixed in 80 per cent. ethyl alcohol for about 8 hours and brought down to distilled water. (Fixation in neutral formalin prolongs the time necessary for decalcification and causes some destruction of phosphatase.)

2. The tissue is placed in a large volume (100 c.cm.) of citrate-HCl buffer at pH 4·5 made up as follows:

Solution A: 21 gm. crystalline citric acid+200 c.cm. N. NaOH per litre.

Solution B: $\dfrac{1}{10}$ N. HCl.

30 c.cm. of B is added to 70 c.cm. of A.

Add 0·2 c.cm. 1 per cent. $ZnSO_4$, and 1 drop of chloroform to prevent the growth of moulds. Keeping the tissues cold (under 10° C.) minimizes the destruction of phosphatase, but delays decalcification. The latter can be hastened considerably by bubbling a stream of air through the fluid.

3. The fluid is changed once or twice daily until decalcification is complete, as indicated by flexibility of the tissue and absence of a precipitate on addition of oxalic acid to the decalcifying fluid. The bones described in this paper took between 2 and 11 days to decalcify, depending on the size and consistency

of the tissue, the fixative, and the decalcifying fluid used. Bones have been kept in citrate buffer for 12 days at 8° C. without adverse effect on their phosphatase content.

4. The tissue is washed in tap-water for several hours.

5. It is then reactivated in 1 per cent. sodium barbitone containing 0·075 per cent. glycine at 37° C. for 3–6 hours.

6. The reactivator is removed by washing in running water for 2–4 hours. (This is important as glycine interferes with the precipitation of calcium phosphate (Fell and Danielli, 1943).)

7. The tissue is dehydrated, cleared, and embedded in wax (m.p. 58° C.).

8. Sections are cut at 8μ, mounted, and brought to distilled water.

9. Slides are incubated with Gomori's substrate at 37° C. The following slightly modified substrate mixture was used:

> 10 c.cm. 2 per cent. calcium nitrate.
> 10 c.cm. 2 per cent. magnesium chloride.
> 10 c.cm. 4 per cent. sodium β-glycerophosphate.
> 70 c.cm. 1 per cent. sodium barbitone.

Incubation times were varied from 20 minutes to 15 hours according to the nature of the material.

The slides are then washed in 1 per cent. calcium nitrate, placed in 2 per cent. cobalt nitrate, washed in distilled water, placed in dilute ammonium sulphide, washed, dehydrated, cleared, and mounted.

The precautions recommended by Danielli (1946) regarding the times spent in the various reagents were observed throughout. Phosphatase sites appear black, the rest of the section being colourless.

10. The section may be counterstained with eosin.

The following point concerning the Gomori–Takamatsu (1939) method may be worth mentioning: cartilage often stains 'positive' in incubated as well as control sections. Bourne (1943b) states that this is of no significance, yet in thick sections (e.g. 30 μ sections of rib as used by Bourne) this effect would mask the presence of phosphatase as well as preformed phosphate if such were present. Actually the positive stain is due to adsorption of cobalt nitrate and ammonium sulphide by the cartilage matrix. It is not abolished by decalcification, but 8 μ sections do not show appreciable staining. If the silver modification of Gomori's technique is used the cartilage remains completely colourless in phosphatase-free areas.

In order to ascertain whether there was any destruction of phosphatase during the process of decalcification a piece of kidney was fixed simultaneously with each piece of bone. It was then divided into two halves, one half to be taken through the same process as the bone and the other to be dehydrated, cleared, and embedded immediately. By comparing sections from the two blocks of kidney mounted on the same slide as the bone section and treated identically, the degree of destruction of phosphatase could be roughly ascertained. As a rule there was very little difference between the kidney sections.
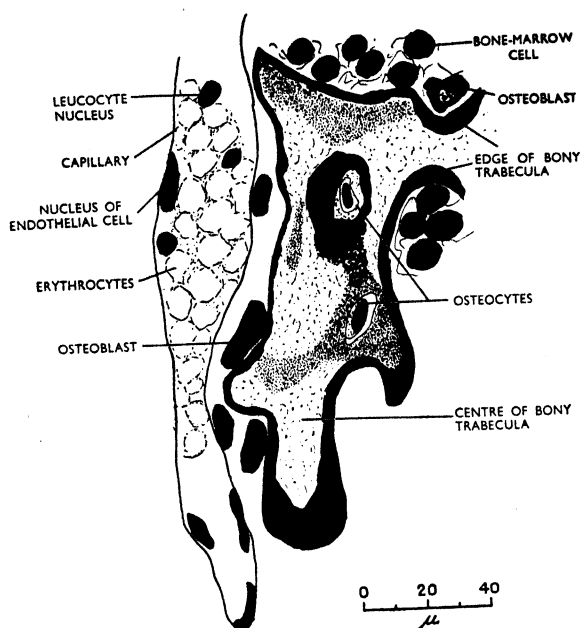
TEXT-FIG. 1.   High-power view of a trabecula.   From the spongy bone close to the costo-chondral junction of the rib of a new-born kitten.

All sections are from decalcified bones cut at 8μ.   Phosphatase is visualized by the Gomori method.   Sites of phosphatase activity appear black.   The drawings were made with the aid of a camera lucida.
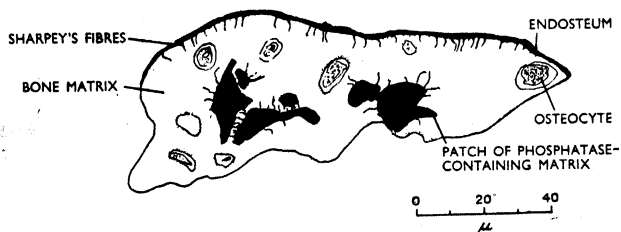
TEXT-FIG. 2.   A bony trabecula near the costo-chondral junction of the rib of an adult rat.

It is assumed that bone phosphatase would be affected by the reagents to the same degree as kidney phosphatase.

RESULTS

The three types of bone—ribs, long bones, and membrane bone—will be described separately.

1. *Ribs*

(a) *From new-born kitten* (Pl. 1, fig. 1, and Text-fig. 1). At some distance from the costo-chondral junction the cartilage is completely negative. Near

TEXT-FIG. 3. From the zone of hypertrophic cartilage at the costo-chondral junction of the rat's rib. Eleven days in acetate buffer. Incubation time 20 min. Note the strongly positive nuclei and the 'capsules' of phosphatase-containing matrix.

the junction the chondrocyte nuclei in the centre of the cartilage stain faintly, those at the periphery more strongly. The matrix is still negative. The peri-chondrium in the region of the columns of cartilage cells is strongly positive and appears as a homogeneous black band in sections incubated for long periods. Shorter incubation times show that the fibroblast nuclei as well as the fibres of the perichondrium contain phosphatase. Two layers can be distinguished in the perichondrium: an inner strongly positive layer and an outer faintly staining one.

The hypertrophic cartilage cells at the costo-chondral junction are free from phosphatase and there are only traces in the matrix. There is an abrupt transition from the colourless cartilage to the heavily staining zone of vascular spongy bone. Here the concentration of phosphatase is greatest at the edges of the bony trabeculae and in the matrix immediately surrounding the osteo-cytes. Osteoblast nuclei and the osteocytes of the newly formed trabeculae are positive. The bone marrow cells are strongly positive. The older osteo-cytes in the centre of larger trabeculae are negative. Trabeculae somewhat removed from the junction show phosphatase only in the matrix immediately

surrounding the osteocytes. The periosteal bone matrix is negative. Only the lining of the vascular canals and a few osteocytes in their vicinity stain black.

TEXT-FIG. 4. Compact bone of rat's rib near the marrow cavity. Eleven days in acetate buffer. Incubation time 1 hour. No counterstain. Note osteocytes with processes and the positively staining fibres extending from the endosteum.

TEXT-FIG. 5. Longitudinal section through the head of the humerus of an adult rat. The marrow cavity is not cut. Four days in citrate buffer. Incubation time 1 hour. Counterstained with eosin. Note the strongly positive Haversian canals, bone marrow, endosteum, periosteum, and hypertrophic cartilage zone. The small-celled cartilage and the bone matrix are negative.

(b) *From adult rat* (Pl. 1, fig. 2, and Text-fig. 3). The costal cartilage including the perichondrium is free from phosphatase except for the hypertrophied cells at the costo-chondral junction which display strongly positive nuclei. The ground substance in this region is also positive. The inner layer

of the perichondrium is positive only at the point where it merges into the periosteum. The latter is strongly positive throughout. The appearance of the trabecular bone does not differ greatly from that in the kitten rib but the phosphatase in the matrix of the newly formed trabeculae appears in patches rather than in the form of a 'halo' round the osteocytes (Text-fig. 2).

The endosteum appears as a black line round each individual fragment of bone. Fine black fibres are seen to enter the bone matrix from the endosteum
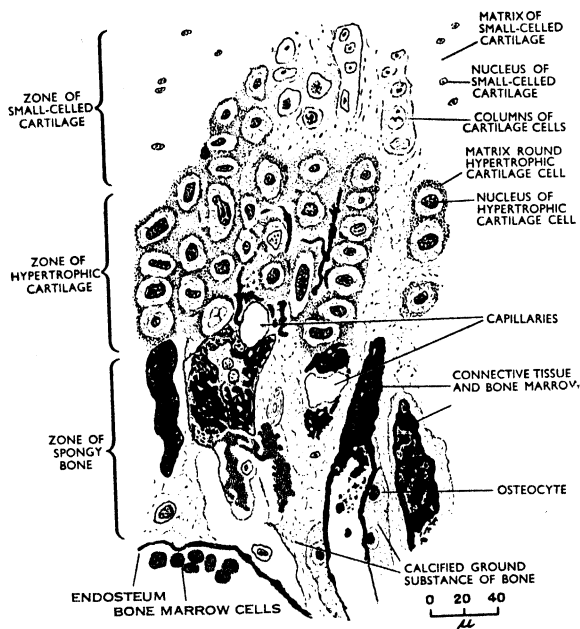
TEXT-FIG. 6. From the epiphyseal junction of the rat femur shown in Text-fig. 5.

(Sharpey's fibres). This is particularly clear in the compact layer of periosteal bone which also displays positively staining osteocytes near the marrow cavity (Text-fig. 4). These osteocytes with their extensively branching processes stand out well against the colourless (i.e. negative) bone matrix.

2. *Long Bones*

(a) *Rat and Mouse.* In the rat and mouse bones examined epiphyseal union had not taken place. This is in accordance with results obtained by Dawson (1925) who showed that the cartilaginous plate persists in the humeri and femurs even of very old rats. The results described refer to the head of the humerus of a fully grown rat (Text-fig. 5) but apply equally to other bones of rats and mice. In the trabecular bone of the epiphysis only the endosteum and the bone marrow contain phosphatase while the matrix is negative. The

diaphysis shows a layer of compact bone in which the Haversian canals stand out black. No osteocytes are stained in the older bone but in the trabeculae near the epiphyseal plate occasional cells with their processes are picked out by the reaction. Sharpey's fibres are clearly seen (cf. Text-fig. 2).

TEXT-FIG. 7. From the costal cartilage and adjacent bone of the rat's femur. Five days in citrate buffer. Incubation time 1 hour. No counterstain. Compare the appearance of the cartilage cells with Text-fig. 3. The endosteum and the nuclei in its vicinity are positive.

TEXT-FIG. 8. Vertical section through the frontal of a new-born kitten. Three days in acetate buffer. Incubation time 1 hour. Counterstained with eosin. Upper edge of section represents outer surface of bone. Note rows of positively staining osteoblasts especially at the outer edges of the bone. The multinucleated osteoblasts at the inner surface are in regions of low phosphatase activity. The framed areas are shown under higher magnification in Text-figs. 9 and 10.

The small-celled cartilage of the epiphyseal plate is negative (Text-fig. 6). The nuclei of the hypertrophic cells are strongly positive and the matrix moderately so. At the transition of the small-celled cartilage to the trabecular bone of the epiphysis occasional enlarged cartilage cells are seen. These have positive nuclei and are surrounded by phosphatase-containing capsules. The

cells of the articular cartilage also have heavily staining nuclei and their capsules are lined with phosphatase (Text-fig. 7). There are a few patches of extracellular phosphatase. In the rat bones only the inner layer of the articular cartilage gives a positive reaction, whereas in the mouse bones the nuclei are strongly positive throughout its depth. The perichondrium is negative. In the diaphysis the fibres and cells of the inner layer of the periosteum give a marked reaction. The periosteum of the epiphysis is negative as is also the small area of perichondrium at the epiphyseal junction.

At the insertion of the tendon there is a gradual transition from fibro-cartilage to tendon tissue (Text-fig. 5). The cells of the former are arranged
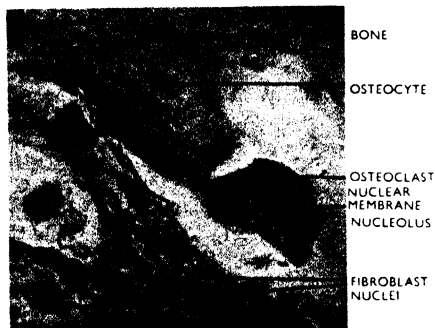
TEXT-FIG. 9. High-power view of an osteoclast and surrounding tissues. Only the nucleoli and the nuclear membrane are positive.

in parallel rows. Their size decreases until they finally represent tendon cells. The large cartilage cells and their capsules stain heavily. The nucleoli and nuclear membranes of the tendon cells stain faintly.

The bone marrow is not well enough preserved to distinguish the cell types. About 75 per cent. of the small cells are strongly positive. The megaloplaxes are negative.

(b) *Kitten* (proximal end of femur: Pl. 1, fig. 3, see p. 381). Here the epiphysis is completely cartilaginous, no secondary centre of ossification having appeared. The small-celled cartilage is devoid of phosphatase. The hypertrophic cartilage cells have strongly positive nuclei and some of the capsules are well marked by black granular deposits. The concentration of phosphatase in the hypertrophic zone is, however, low in comparison with that in the bone marrow of the invading spongy bone. The trabeculae nearest the eroding zone are faintly positive at the edges, those farther away are negative. Numerous phosphatase containing osteoblasts similar in appearance to those shown in Text-fig. 10 are seen along the trabeculae. The fibroblast nuclei and fibres of the periosteum are positive. The endosteum is not as clearly defined as was the case in the rodent bones.

3. Membrane Bone

(a) *Rat and Mouse.* The compact bone is identical in appearance with that seen in the cartilage bones, i.e. the matrix is free from phosphatase while the Haversian canals and isolated osteocytes near them stain black.

The periosteum on the external (skin) side is positive while that of the internal surface is mostly negative. The strands of connective tissue penetrating the bone or uniting two adjacent bones are positive only in the layer close to the bony surface. More marrow spaces were found in the mouse than in the rat skull. They are lined by positively staining endosteum. The bone marrow again stains intensely.

TEXT-FIG. 10. High-power view of osteoblasts lining a bony trabecula. Note the presence of phosphatase in the cytoplasm as well as the nuclei, and in the connective tissue fibrils. The bone matrix is faintly positive.

(b) *Kitten* (vertical section through the frontal: Text-figs. 8, 9, and 10). Here membrane bone formation is still in progress. The appearance of the trabeculae and osteoblasts is similar to that in the long bones. Osteoclasts which only display positive nucleoli and nuclear membranes (Text-fig. 9) are often observed at the surface near the inner (brain) edge of the bone while osteoblasts occur mainly at the opposite side. Most of the connective tissue-cell nuclei are moderately positive, more so in the immediate vicinity of a trabecula. In general the concentration of phosphatase is greater towards the skin side, i.e. where the majority of osteoblasts are congregated. Very fine black fibrils may be seen in the connective tissue particularly near osteoblasts (Text-fig. 10). In some places these fibrils appear to penetrate the newly formed trabeculae.

DISCUSSION

The number of bones examined being very limited, only preliminary conclusions can be drawn. The following points emerge from a consideration of the adult rodent bones as well as the kitten bones: phosphatase is concentrated in the endosteum and the periosteum where it appears in fibroblast nuclei and

on the collagenous fibres. This agrees with results obtained by Gomori (1943) and Bourne (1943), although the latter attributes the positive reaction of the periosteum to the capillaries and osteoblasts. The bone matrix itself contains no phosphatase except in some newly formed trabeculae where traces of the enzyme were detected. This could not previously be shown on account of the interference of preformed phosphate. As Bourne (1943) has noted, the superficially placed osteocytes give a positive reaction. By the present method these cells are shown to contain phosphatase not only in the cell-body, but also in the fine ramifications which spread out into the bone matrix. The lining of the Haversian canals of compact bone is invariably positive as also is that of the marrow spaces and the cells contained therein. It seems likely that the high concentration of phosphatase in these vascular channels assures a constantly high level of inorganic phosphate in any tissue or tissue fluid coming in contact with the bone matrix, with which it must be in equilibrium.

The absence of phosphatase from the hypertrophic cartilage cells in the kitten ribs is surprising and should be confirmed by further work. In the cartilage bones of the rodents the zone of hypertrophied cartilage is positive, there being some extracellular as well as high concentrations of nuclear phosphatase. Osteoblasts are strongly positive wherever they occur, but this fact alone does not constitute proof of Fell and Robison's (1929, 1930) conclusion that bone phosphatase is secreted by the osteoblasts and hypertrophic cartilage cells. As has been shown, most of the bone marrow cells and the perichondral fibroblasts contain equally high concentrations of the enzyme. What does seem significant, however, is the fact that in zones of active deposition of ground substance a certain amount of extracellular phosphatase is always found. This is noticeable in the zone of hypertrophic cartilage of the rodent long bones as well as in the kitten skull where the osteoblasts seem to be surrounded by a web of positive fibres. At points where presumably reabsorption of bone is taking place as indicated by the presence of osteoclasts, there is no extracellular phosphatase although the nuclei of many mesenchymal cells are positive. Danielli (1946) has suggested that phosphatase might only be active (*a*) in the cell nucleus, (*b*) outside the cell, the conditions of pH and SH in the cytoplasm being unfavourable. The above results seem to support this theory, as newly deposited bone-salts are never observed in areas where there is no extracellular phosphatase. Nuclear phosphatase may be concerned with nucleic acid metabolism (Krugelis, 1946). In any case it is not correlated with the appearance of calcification. Yet although it seems impossible for bone-salt to be deposited in normal tissues in the absence of extracellular phosphatase the converse is not true: phosphatase is found on the fibres of the periosteum which is not itself calcified, and in the fibres of healing wounds where it is supposed to play a part in the formation of collagen fibres (Fell and Danielli, 1943). The failure of these tissues to calcify in spite of the presence of extracellular phosphatase may be related to a lack of Robison's '2nd factor' (Robison, 1930), a condition which makes calcification

impossible. Further research into the nature and distribution of this '2nd factor' is needed before the functions of phosphatase can be fully understood.

SUMMARY

1. Adult mammalian bones may be decalcified in sodium citrate-HCl buffer at pH 4·5. The addition of $Zn^{..}$ (10^{-4} M) prevents irreversible destruction of alkaline phosphatase which is, however, partially inactivated.

2. The phosphatase is subsequently reactivated by placing the tissues in 1 per cent. sodium barbitone+0·075 per cent. glycine at 37° C. for 2–3 hours.

3. In this way thin paraffin sections free from preformed phosphate are obtained.

4. Phosphatase is demonstrated by the Gomori–Takamatsu (1939) technique.

5. Long bones, ribs, and membrane bone from the skull of adult rats and mice and of new-born kittens were examined.

6. Phosphatase was found to be present in the endosteum, the inner layer of the periosteum, the Haversian canals and linings of vascular and marrow spaces, the nuclei of bone marrow and connective tissue-cells, and in isolated osteocytes. The hypertrophic cartilage was positive in both nuclei and matrix in the rodent bones but negative in the kitten bones. The bone matrix and the small-celled cartilage were negative throughout.

7. The possible significance of these findings in relation to the function of alkaline phosphatase is discussed.

POSTSCRIPT

A paper describing a decalcification technique not unlike the one here proposed has recently been published (Greep, Fischer, and Morse, 1947). The authors propose the use of citrate solutions at pH 4·8 to 5·0. They state that a slight increase in acidity leads to irreversible loss of phosphatase activity. The present paper shows that in the presence of $Zn^{..}$ ions solutions at pH 4·5 can safely be used. Greep *et al.* suggest that solutions used for washing and dehydrating the tissues after decalcification should be buffered at pH 9·3 'in order to provide immediately an optimum condition for the phosphatase to retain and *perhaps regain* activity' (my italics). The fact that phosphatase can indeed be reactivated by alkaline solutions, especially in the presence of amino acids, has formed the basis of my decalcification method and has now been independently confirmed by the work of Greep, Fischer, and Morse.

DESCRIPTION OF PLATE I

All sections are from decalcified bones and are cut at 8 μ. Phosphatase is visualized by Gomori's method. Sites of phosphatase activity appear black. Cartilage is seen on the right of the section, bone on the left.

Plate 1, fig. 1. Costo-chondral junction from rib of new-born kitten. Two days in citrate buffer. Incubation time 15 hours. No counterstain. Note the absence of phosphatase from the small-celled cartilage including the hypertrophied zone, and the strongly positive vascular zone of spongy bone (on left half of the photograph). The edges of the bony trabeculae are positive. Phosphatase containing leucocytes are seen in the blood-vessels. The erythrocytes are negative.

Plate 1, fig. 2. Costo-chondral junction from rib of adult rat. Six days in acetate buffer. Incubation time 30 minutes. No counterstain. Note the absence of phosphatase from the small-celled cartilage except a few nuclei in the central zone. Nuclei of hypertrophic cartilage cells are strongly positive. The inner layer of the periosteum is positive, the compact periosteal bone immediately below it is negative. There are a few positive patches in the bony trabeculae.

Plate 1, fig. 3. Longitudinal section through the proximal end of the femur of a new-born kitten, showing the epiphysial junction. Seven days in acetate buffer. Incubation time 45 minutes. Counterstained with eosin. Note phosphatase in nuclei and in some 'capsules' of the hypertrophied cartilage cells in the inner layer of the periosteum and in the marrow spaces. The periosteal bone is negative.

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BONY
TRABECULA

BLOOD
VESSEL

100μ

SMALL-CELLED
CARTILAGE

HYPERTROPHIC
CARTILAGE

PERIOSTEUM

FIG. 1

OUTER LAYER
OF PERIOSTEUM

INNER LAYER OF
PERIOSTEUM

PERIOSTEAL BONE

BONE MARROW

BONY TRABECULAE

SMALL-CELLED
CARTILAGE

HYPERTROPHIC
CARTILAGE

BLOOD VESSEL

100μ   FIG. 2

100μ

PERIOSTEUM

PERIOSTEAL BONE

BONY TRABECULAE

HYPERTROPHIC
CARTILAGE

BLOOD VESSEL

BONE MARROW
AND OSTEOBLASTS

EPIPHYSEAL
CARTILAGE

FIG. 3

The Use of Nile Blue in the Examination of Lipoids

BY

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CONTENTS

INTRODUCTION

NILE blue was introduced by Lorrain Smith (1908) for distinguishing neutral fats (triglycerides) from fatty acids, the former being coloured red, the latter dark blue. Kaufmann and Lehmann (1926) applied the dye to a large number of mixtures of lipoids, and concluded that it was of no histochemical value at all. Lison (1935 *a* and *b*), after a profound study, concluded that the red coloration was characteristic of lipoids in general, and the blue was merely that of a basic dye and therefore totally unspecific. These conclusions supersede those in his book (1936).

This paper presents evidence that if a substance is known beforehand to be lipoid and colours red with nile blue, it consists of neutral lipoids (esters and/or hydrocarbons); if it colours blue, it may contain these, but acidic lipoids (fatty acids, phospholipines, and perhaps some others) are certainly present as well. The presence or absence of cholesterol cannot be established with nile blue.

The term 'nile blue' is used throughout to mean the commercial dye, which, as Lison has shown, is a mixture of the oxazine sulphate (true nile blue) and the oxazone (nile red).

MATERIALS AND METHODS

The tissues and cells examined were: adipose tissue and adrenal cortex of the rat, adipose tissue of the mouse, sebaceous glands in guinea-pig skin, and

fat-cells and stomach epithelium of the Rhynchobdellid leech *Glossiphonia complanata* (L.).

Formal-calcium fixation was always used, generally with postchroming as for Baker's acid haematein test for lipines (1946). Sections were cut on the freezing microtome, stained in a 1 per cent. aqueous solution of nile blue for 5 minutes, differentiated in 1 per cent. acetic acid for 30 seconds, washed, and mounted in Farrants's medium. In certain cases a 0·02 per cent. solution of nile blue was used.

Experiments were also carried out on various lipoids *in vitro*; these are described below.

RESULTS AND DISCUSSION

I. *Observations on tissues*

It was observed that

(*a*) the fat droplets in the cells of rat and mouse adipose tissue always coloured red with 1 per cent. nile blue,

(*b*) the lipoid droplets in rat adrenal cortex cells (Harrison and Cain, 1947) and in the fat-cells of *Glossiphonia* might be red, blue, or some intermediate colour,

(*c*) the contents of guinea-pig sebaceous glands were always red, and

(*d*) the lipoid component of the Golgi body in stomach-epithelial cells of *Glossiphonia* stained deep blue, but went red after very prolonged differentiation (Cain 1947).

There is, then, a definite phenomenon to be investigated.

II. *Examination of Kaufmann and Lehmann's results*

(*a*) *General considerations.* Lison (1935*b*) concluded that the red substance that colours lipoids was not the free base of nile blue, as had been thought, but an oxidation product, an oxazone, which he named 'nile red'. Smith had shown this in his first paper, but his opinion had been disregarded, and he found it necessary to restate his views (1911) and insist that the double coloration was not a case of metachromasy as several authors had assumed.

The reasons why Smith's conclusion was not generally accepted were because (*a*) a red substance was readily extractable from aqueous solutions of nile blue by shaking with toluene or similar solvents, and (*b*) no such substance could be extracted from solid nile blue. Obviously the red substance was produced by hydrolysis, and must be the free base. Lison showed that the substance extractable from 1 per cent. or stronger solutions of nile blue was the oxazone, as Smith had stated, and that, being a very weak base, it formed salts which were insoluble in toluene and were hydrolysed instantly by water. The oxazone was insoluble in water, but soluble to some extent in fairly concentrated solutions of nile blue, and therefore could be extracted with toluene from strong aqueous solutions but not from the solid dye. It behaves like any other lipoid-colorant, such as sudan black or sudan IV, and

cannot be used to distinguish between the various classes of lipoid. Further, he showed that, as Smith had also stated, the free base (oxazine) is obtainable by extracting a concentrated solution of nile blue until all the oxazone is removed, alkalinizing, and extracting again. The solution obtained differs from an oxazone solution in the shade of red, and in being without fluorescence. The base is a strong one and readily combines with atmospheric carbon dioxide and moisture to give the blue carbonate, which, like the sulphate, is soluble in water but not in toluene and similar solvents. The intensity of coloration of the oxazine is far less than that of the oxazone, and it is the latter that colours lipoids in sections.

Lison also showed, for the first time, that whereas 1 per cent. or stronger solutions of nile blue are not hydrolysed, and the only red substance extractable from them is the oxazone, more dilute solutions are hydrolysed, and a mixture of oxazine and oxazone is obtained on extraction. With very dilute solutions almost pure oxazine is obtained, the oxazone being almost insoluble in them. (I have found a 0·02 per cent. solution satisfactory.) On standing, aqueous solutions of oxazine sulphate produce a certain amount of oxazone by oxidation.

He concluded that since the oxazine sulphate was merely a basic dye, and since the oxazone was merely a general lipoid-colorant, the use of the two together gave results of no more significance than would any other comparable combination, and that nile blue should not be used in histochemistry.

Nevertheless, such a combination might be used to distinguish between those lipoid mixtures that do and those that do not contain substances which will take up the blue colour. But Kaufmann and Lehmann (1926) had applied nile blue to a large number of lipoids and mixtures of lipoids and concluded that it was useless. To simulate tissue sections they enclosed the substances to be studied in pith, which was sectioned, stained, and mounted. Lison (1936) comments on their results that (*a*) every time a red colour was obtained, the mixture contained triolein, but many mixtures containing triolein were blue or uncoloured, and (*b*) 31 cases were observed in which mixtures containing free fatty acids (which according to Lorrain Smith should have stained blue) were uncoloured, and in 7 cases mixtures with no fatty acids at all were coloured blue.

The results they obtained with pure lipoids are of great interest. Oleic acid was blue, triolein red. Palmitic and stearic acids, tripalmitin, tristearin, cholesterol, cholesteryl oleate, lecithin (Merck and Kahlbaum), sphingomyelin, phrenosin, and kerasin were all uncoloured. Several of these substances were solid at room temperature, but might well have stained in solution. Others such as lecithin might be expected to stain even in the solid state.

A repetition of some of these results was therefore attempted with small portions smeared or melted on to coverslips. Since triolein was red, they must have used a fairly concentrated solution of nile blue, and a 1 per cent. solution was therefore employed. Tristearin and tripalmitin were

uncoloured, stearic and palmitic acids very faint blue, nearly white, cholesterol uncoloured. All these were solid. Cholesteryl oleate, which was greasy, was pink, and lecithin exceedingly dark blue. It is probable that Kaufmann and Lehmann obtained no result with lecithin because their method of impregnation of pith was not suitable or because it escaped from the sections. On warming tripalmitin with nile blue solution, it coloured red on melting. Triolein and tributyrin, being liquid, coloured red at room temperature.

From these experiments it is seen that Kaufmann and Lehmann's results require reinterpretation. In the first place, lecithin (and therefore perhaps other phospholipines) stains an exceedingly dark blue. In the second place, a negative result may mean merely that the mixture was solid.

(*b*) *Blue-staining mixtures containing triolein.* If the properties of mixtures of lipoids were the sum of the properties of their components, it should be possible to prophesy the result of staining with nile blue from data on pure substances. In view of the blue-staining properties of lecithin and the possibility that fatty acids other than oleic may stain in solution, the apparent exceptions among Kaufmann and Lehmann's results require re-examination. There are 46 cases in which mixtures are not coloured red, although triolein is included. Of these, 35 contained oleic acid (which is known to stain blue) and the result obtained was blue, or in one case *violetblau*. Of the rest, 5 contained lecithin, 1 phrenosin, and 1 kerasin, and all were coloured blue. It is interesting that phrenosin and kerasin seem to behave like lecithin. Baker (1946) noted that galactolipine varied in the response given to his test for lipines, and suggested that pure galactolipine did not respond. It is well known that the complete separation of galactolipine from sphingomyelin is very difficult, and sphingomyelin resembles lecithin and cephalin in containing phosphoric acid. Possibly the samples used by Kaufmann and Lehmann were not quite pure. The remainder of the exceptions are:

(A1) triolein and cholesterol—blue;
(A2) triolein and glycerol—part red, part blue;
(A3) triolein and cholesteryl oleate—*violettros*; and
(A4) triolein, cholesterol, and lecithin—part red, part uncoloured.

The second might have been expected, perhaps, as solid nile blue will dissolve in glycerol to give a blue solution. The third is at least reddish. But the first is wholly unexpected, and obviously requires confirmation.

It was tested in the following ways:

(i) A test-tube was half-filled with 1 per cent. nile blue solution, and some triolein run in. After shaking, the triolein layer was red. Cholesterol crystals were then dissolved in it until it was saturated. Even after prolonged shaking, it remained red, though with a change in tone towards cherry-red. The same result was obtained on repeating the experiment with xylene or tributyrin replacing triolein.

(ii) Nile blue dissolves in acetone, giving a blue solution with a distinct red tinge. Saturation of an acetone-nile blue solution with cholesterol produces no change in colour.

(iii) Addition of cholesterol to a solution of the oxazone in xylene has no effect on the colour.

(iv) Addition of cholesterol to a xylene solution of the free base (oxazine) changes the colour from a clear to a dirty, rather opaque red.

(v) Solid cholesterol, as shown above, does not stain with nile blue. Melted cholesterol colours bright orange-yellow with solid nile blue. As it crystallizes, the colour changes very abruptly to blue.

From these experiments it appears that cholesterol in solution is inert towards nile blue, and melted cholesterol acts only as a solvent. As was expected, there appeared to be no combination. One is forced to the conclusion that at least in this particular case either the cholesterol or the triolein used by Kaufmann and Lehmann was impure, or both were.

The fourth exception given above is also very interesting, as it seems to show that the presence of lecithin is not sufficient to ensure that a mixture will stain blue. In this case, a positive result was obtained with the Smith-Dietrich test at 60° C. and with Schultze's test for cholesterol, so apparently both lecithin and cholesterol were present in the mixture. The five mixtures that contained triolein and lecithin and stained blue were:

(B1) triolein, palmitic acid, and lecithin,
(B2) triolein, stearic acid, and lecithin,
(B3) triolein, stearic acid, cholesterol, and lecithin,
(B4) triolein, tristearin, cholesterol, and lecithin, and
(B5) triolein, cholesterol, glycerol, and lecithin,

the last being coloured blue or purple. The blue coloration of the fourth can only be attributed to lecithin or impurities, but in the case of the first three it is possible that the fatty acids may colour in solution in triolein.

On adding palmitic acid to a xylene solution of oxazone, there was no change of colour. On adding a few c.cm. of 1 per cent. nile blue solution and shaking, the colour of the xylene layer became a reddish purple. The interpretation of this result is that xylene, like triolein, will dissolve from 1 per cent. nile blue only the oxazone, which will not form a salt with a fatty acid. The fatty acid does form a blue salt with the oxazine, and this then dissolves in the xylene layer, turning its colour towards blue. Now Lison (1935*b*) showed that there is very little free oxazine in 1 per cent. nile blue, but a considerable amount in more dilute solutions. Shaking a very dilute solution of nile blue with a xylene solution of palmitic acid should therefore cause the xylene layer to be coloured blue, and this is found to be the case. Addition of a fatty acid to a xylene solution of free oxazine produces an intense blue immediately.

From these results it appears that the staining blue of the first three mixtures (B1, B2, and B3) is more likely to be due to lecithin than to the fatty acids, as Kaufmann and Lehmann used a strong solution of nile blue. The only conclusion possible with respect to mixture A4 above is that the results of the Smith-Dietrich test are not necessarily relevant to the results obtained with nile blue.

Since glycerol gives a blue solution with solid nile blue, it is possible that mixture B5 might be accounted for without invoking lecithin. B4 requires examination, as it contains tristearin.

On dissolving tristearin in xylene or tributyrin solutions of either oxazine or oxazone, no change in colour was produced, nor was any blue imported into the xylene layer on shaking xylene solutions of triglycerides with strong or weak nile blue solutions. Melted triglycerides coloured red with 1 per cent. nile blue, and were almost uncoloured with 0·02 per cent. solutions.

It seems unlikely, then, that the blue colour of B4 can be attributed to anything but lecithin or impurities. The Smith-Dietrich test for this mixture gave a positive result, but so it did for mixture A4.

(c) *Red-coloured mixtures containing triolein.* The mixture (A4) of triolein, cholesterol, and lecithin which was partly red and partly uncoloured has already been mentioned. On a mixture of triolein and lecithin which coloured red Kaufmann and Lehmann comment *blaszt bald ab*, yet this mixture gave a positive result with the Smith-Dietrich test at 60° C. and therefore might have been expected to stain blue. It seems impossible to correlate the results of different tests upon the same mixture.

On adding lecithin to a xylene layer over 1 per cent. nile blue and shaking, the xylene went from red to blue. On dissolving lecithin in a xylene solution of oxazone, there was no change in colour, but with an oxazine solution there was an immediate change to blue.

Apart from the two mixtures just mentioned, ten cases of red coloration of mixtures containing triolein, exclusive of those giving purple or other intermediate tones, were observed by Kaufmann and Lehmann. Of these, only one (C9 below) contained oleic acid. They were:

(C1) triolein and palmitic acid,
(C2) triolein and stearic acid,
(C3) triolein, palmitic acid, and cholesterol,
(C4) triolein, stearic acid, and cholesterol,
(C5) triolein and sphingomyelin,
(C6) triolein and glycerol (part red, part blue),
(C7) triolein, stearic acid, and glycerol,
(C8) triolein and albumen,
(C9) triolein, kerasin, and oleic acid,
(C10) triolein and cholesteryl stearate.

The first four are explicable if the mixtures were tested with a strong solution of nile blue. The tenth is to be expected if cholesteryl esters behave like triglycerides.

On dissolving cholesteryl acetate or stearate in solutions of oxazone or oxazine in xylene, no change in colour was produced. These esters do behave like triglycerides.

The rest, except C9, are not surprising, but as confirmatory experiments on sphingomyelin, phrenosin, and kerasin could not be done, no definite comments on them can be made. The only explanation for C9 is that there must have been very little oleic acid present.

No mixtures were coloured red that did not contain triolein.

(*d*) *Other mixtures, blue-stained or uncoloured.* Forty-two mixtures are stated to have been uncoloured, one is recorded as fugitive, and two (mentioned above) as occasionally uncoloured. Such mixtures as tripalmitin and palmitic acid, tripalmitin and stearic acid, palmitic acid and tristearin, palmitic acid and cholesterol, or tristearin and cholesterol one would expect to be uncoloured, as they are solid at room temperatures. Others are more surprising. Such mixtures as palmitic acid and lecithin, stearic acid and lecithin, tripalmitin and lecithin, and stearic acid with tristearin and lecithin, might be expected to stain at least superficially. Again, in the absence of confirmatory observations on pure sphingomyelin and galactolipines it is not possible to comment on some mixtures.

Considering only mixtures containing fatty acids, in twenty-three cases in which there was no coloration it is likely that the mixture was solid and could not take up the stain. In only one case, a mixture of oleic acid and kerasin, was there no coloration when it might reasonably have been expected. This same mixture gave a positive result with the Smith-Dietrich test at 60° C.

III. *Discussion*

It is seen from the above examination of Kaufmann and Lehmann's results, and the supplementary experiments, that in one case at least there is doubt as to the purity of the substances used by these workers, and that correlation of the results obtained by different tests on the same mixture is difficult. Most of the results, with some exceptions (one of which is directly contradicted by *in vitro* experiment) are consonant with the following propositions:

(*a*) Substances in the solid state (except greases) do not colour in any way with aqueous solutions of nile blue.

(*b*) Triglycerides, liquid or dissolved in hydrocarbons or other triglycerides, are coloured red, if pure, by the oxazone or, much less intensely, by the free base (oxazine).

(*c*) Fatty acids if liquid colour blue with solutions of oxazine or dilute nile blue solutions. With strong solutions of nile blue, only a slight change towards blue is seen except with oleic acid which colours fairly strongly.

(d) Lecithin (and perhaps all phospholipines) stains deep blue when solid. It has no effect on oxazone solutions, turns oxazine solutions blue, and colours blue with weak or strong aqueous solutions of nile blue.

(e) Cholesterol in solution (in fat-solvents) is inert towards nile blue, and the solution will not dissolve nile blue sulphate.

In connexion with the last proposition it is interesting to note that acetone and ethyl alcohol will dissolve the sulphate, but terpineol behaves like cholesterol and its esters, triglycerides, xylene, toluene, benzene, carbon disulphide, and pinene.

The principles underlying these propositions are that neutral lipoids will dissolve out of aqueous solutions of nile blue only the oxazone and free base (the latter not being present in appreciable quantities in 1 per cent. solution) but acidic lipoids will dissolve the oxazone and combine with the free base to give blue compounds which are soluble in lipoids and may mask the red coloration due to the oxazone. Alcohols act as solvents, the water-soluble ones dissolving oxazine, oxazine sulphate, and oxazone, the other dissolving only the oxazine and oxazone.

It will be noted that only lecithin and oleic acid will colour blue to any great extent with 1 per cent. solutions of nile blue. Oleic acid is of the order of strength of stearic and palmitic acids, but these require the free base in quantity to colour well. In view of the intersolubility of lipoids, the great difficulty of preparing them pure except by synthesis, and the natural origin of most oleic acid, it is possible that the samples used contained a certain amount of lipine and so could colour blue even with 1 per cent. solutions. Alternatively, it is just possible that a small amount of oxazine sulphate can dissolve in oleic acid and so colour it. Certainly, the intensity of staining of lecithin appears much greater than that of oleic acid, which makes it probable that lipoids staining blue in tissue sections with 1 per cent. nile blue contain lipine. However, this cannot be used yet to give histochemical conclusions.

To make full use of nile blue one must employ both the oxazone and the oxazine. The former is the intense colorant for lipoids, the latter the reagent for fatty acids. Further, the lipoids under investigation must be liquid. The following procedure is suggested:

(i) Fix in formal-calcium, with or without postchroming.

(ii) Cut sections on the freezing microtome.

(iii) Colour one section in sudan black, to define the lipoids present.

(iv) Stain one section in 1 per cent. nile blue at 60° C. or at 37° C., for 5 minutes, wash quickly in water at the same temperature, and differentiate in 1 per cent. acetic acid for 30 seconds, still at the same temperature. (It is necessary to work at the same temperature throughout, otherwise some lipoid may entrap a small amount of stain in crystallizing, and this will not be removed by washing and differentiating.)

(v) Mount in Farrants's or some similar medium. This section will show what lipoids will colour blue with 1 per cent. nile blue.

(vi) Repeat with another section and immediately after staining restain in a 0·02 per cent. solution of nile blue at the same temperature, then wash, differentiate, and mount as before. Comparison of the two nile blue sections should then indicate where in the tissues fatty acid is to be found, if there is any other than oleic acid present.

If there is no observable difference between the two sections, then the first may be dispensed with, as what will stain blue with 1 per cent. nile blue will stain with the 0·02 per cent. solution.

There are certain points to be noted when interpreting results. In the first place, only purely lipoid inclusions can be considered. Nile blue sulphate is a basic dye, as Lison has reminded us, and is not in the least specific for lipoids. It will stain most elements of a tissue, and it is not possible to distinguish between blue-stained lipoids and other substances by prolonged differentiation. It is true that lecithin is still deep blue after 24 hours' differentiation, which may be useful at times as an indication, but some acidic proteins are also still stained after this period. There is still a faint blue in nuclei, for example. It is not permissible to prepare sections for Baker's acid haematein and pyridine extraction tests, stain them in nile blue, and compare them. The pyridine, perhaps because it is a strong base, has a definite depressant effect on staining by nile blue sulphate in regions where no lipoid can be shown by sudan black. The only safe method for determining whether a body is purely lipoid is to colour pyridine-extracted sections with sudan black and see if it has been completely removed. If not, no conclusion can be drawn from results obtained with nile blue, unless it can be shown that the residue after extraction with some other lipoid-solvent will not stain blue, or will do so only with markedly reduced intensity. Also, if a body will colour only red with nile blue, it does not necessarily mean that acidic lipoids are completely absent. But, of course, if a blue-stained body can be turned red by prolonged differentiation, that is proof that lipoid is present. This method has been used (Cain, 1947) to apply the oxazone in an aqueous medium to a structure in which the use of the enormously powerful and opaque sudan black showed no details at all. The oxazone did not render the body opaque, and so revealed some details of its construction. Finally, such lipoid inclusions as cholesterol crystals will not liquefy at 60° C. and cannot be examined with nile blue.

My thanks are due to Dr. J. R. Baker, who has supervised the work set out in this paper.

SUMMARY

Nile blue, introduced by Lorrain Smith for distinguishing between neutral fats (triglycerides) and fatty acids, was considered by Lison to be of no histochemical value except that the red coloration was specific for lipoids in

general. This conclusion was based principally on Lison's own researches, and in part on those of Kaufmann and Lehmann.

Reinvestigation has shown that some of Kaufmann and Lehmann's results are of doubtful value and all require careful interpretation, but in general these and other results lead to the conclusion that nile blue can be used to distinguish neutral lipoids (esters and hydrocarbons) from acidic lipoids (phospholipines and acids). Cholesterol is not detected.

A method for using both the oxazine and oxazone of nile blue is described, which has greater sensitivity to acidic lipoids.

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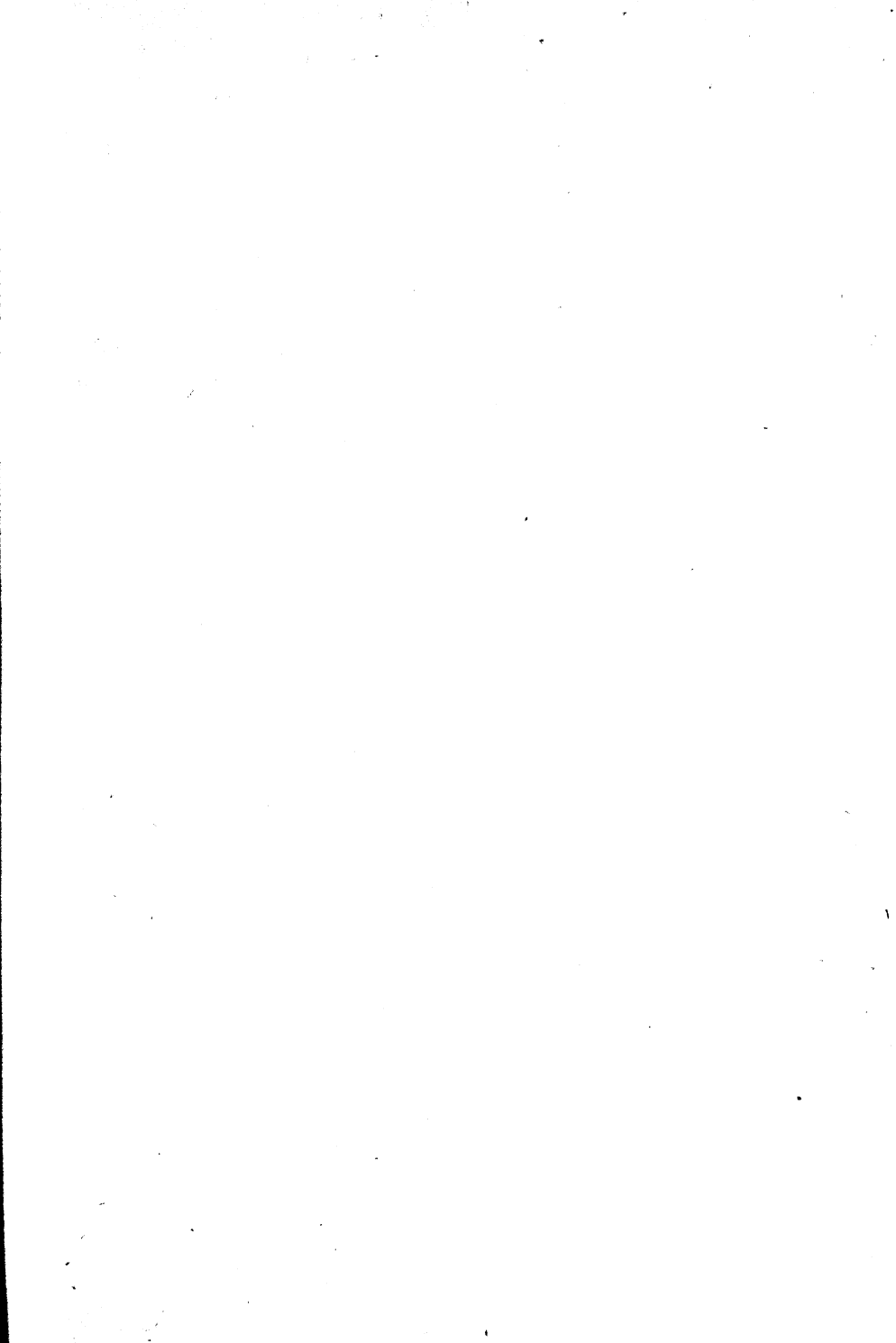
The Developmental Cycle of Botrylloides

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With five Text-figures

O F the many studies made on ascidians in the past, few have included the organism in its entirety. The present account is of the whole developmental cycle of an organism exhibiting exquisite timing in its four-dimensional relationships, with an interpretation of a colonial organism as the result of a few interacting processes. A comparison of *Botrylloides* with *Botryllus* affords some analysis of the nature of differences of generic magnitude, as modifications of several basic activities.

Botrylloides is an ascidian so clearly related to *Botryllus* that it has often been relegated to the status of a subgenus of that form. Only a few species are known and species differences are very small indeed. The type discussed here is *Botrylloides leachi* Savigny; others are *B. diegense* of the North American Pacific coast, stated by Van Name (1945, p. 230) to be an extremely close ally of *B. leachi*, *B. aureum*, a more northern form differing mainly in the position of the sperm duct opening and in colour range, and *B. nigrum* of the West Indies that personal investigation has shown to be essentially the same as *B. leachi* except for colour range. Species of the genus *Botryllus* are almost equally restricted in number and order of differences, except that in two species the number of rows of stigmata is reduced to four. We can accordingly speak in the more general terms of reference to the genus *Botrylloides* and the genus *Botryllus* and to the nature and origin of the generic differences between them. Most of the peculiarities of *Botrylloides* represent specializations of features present in *Botryllus*, and with some reserve the assumption is made that there has been an actual evolution from *Botryllus* itself, the latter showing little evidence of independent specializations of its own, and there seems to be no need to introduce a hypothetical common ancestor significantly different from it.

STRUCTURE OF ZOOID

The zooid structure is in general similar to that of *Botryllus* zooids and, apart from the nature of the atrial siphon, very much like zooids of *Symplegma* (Berrill, 1940). In all three the branchial sac is secondarily simple, the four folds typical of the Polystylidae being reduced to three, and each fold represented by a single internal longitudinal vessel. Likewise the gonads, characteristically scattered as polycarps over the atrial wall, are present as a single

hermaphrodite unit on each side. The branchial sac opens into the looped digestive tube, of which the stomach possesses longitudinal hepatic folds. The heart is compact and lies on the opposite side close to the base of the endostyle. The peribranchial sacs, diverging from the atrial chamber along the left and right sides of the branchial sac, extend beyond the abdomen to meet posteriorly, though without fusing. In consequence, all the visceral organs are separated from circulating sea-water by a simple epithelial membrane.

The distinctive features of the *Botrylloides* zooid are the more elongate shape of the branchial sac, due to relatively more rows of stigmata and fewer stigmata per row, the wide atrial siphon, the orientation of the gonad, the ovary being ventral instead of lateral to the testes, and the placental connexion with the solitary developing embryo on each side (fig. 1 B, C).

ATRIAL SIPHON AND THE FORMATION OF SYSTEMS

In *Symplegma* atrial siphons are not hypertrophied relative to the branchial siphons, and no systems are formed. In *Botryllus* the atrial siphons are enlarged and conical, and buds developing adjacently to one another orientate in the final stages to form typical star-shaped systems, the narrow ends of the atrial cones opening into the common cloacal chamber of each system. In *Botrylloides* the atrial siphon exhibits a greater range of form. In very small individuals, of the first few asexual generations, the atrial siphons are no larger than the branchial, and no systems are formed. When somewhat larger, conical siphons develop and there is a tendency to produce star-systems as in *Botryllus*. In mature *Botrylloides* colonies, however, two types coexist, square-edged forms that can inevitably form only ladder-like systems, and conical forms that comprise the ends of the separate ladder systems. The two together account both for the existence of the ladder system and its limits, while the progressive change in size and form of the atrial siphon seems to be a typical case of relative growth (fig. 1 A).

BUDDING CYCLE

Buds are formed in *Botrylloides* at the same place and stage as in *Botryllus*. At the stage when stigmata are visible merely as imperforate rows, a bud disk appears on each side mainly involving the atrial epithelium. When the parent bud is almost half-grown, its buds already exhibit the principal morphological divisions, namely, the branchial, peribranchial, neural, and ovarian units. When fully grown and just functioning, a zooid bears buds that are a little younger than the stage at which the subsequent bud generation appears. In the final phase of bud growth, which is primarily one of cell growth and differentiation, in contrast to cell proliferation, the bud draws so heavily on the nutritional supply from its parent that the latter undergoes resorption (fig. 1 B).

There is accordingly a very definite cycle of bud initiation, development, and growth, followed by a period of survival as a functional organism, ending when the buds of the next generation attain in turn their functional maturity.
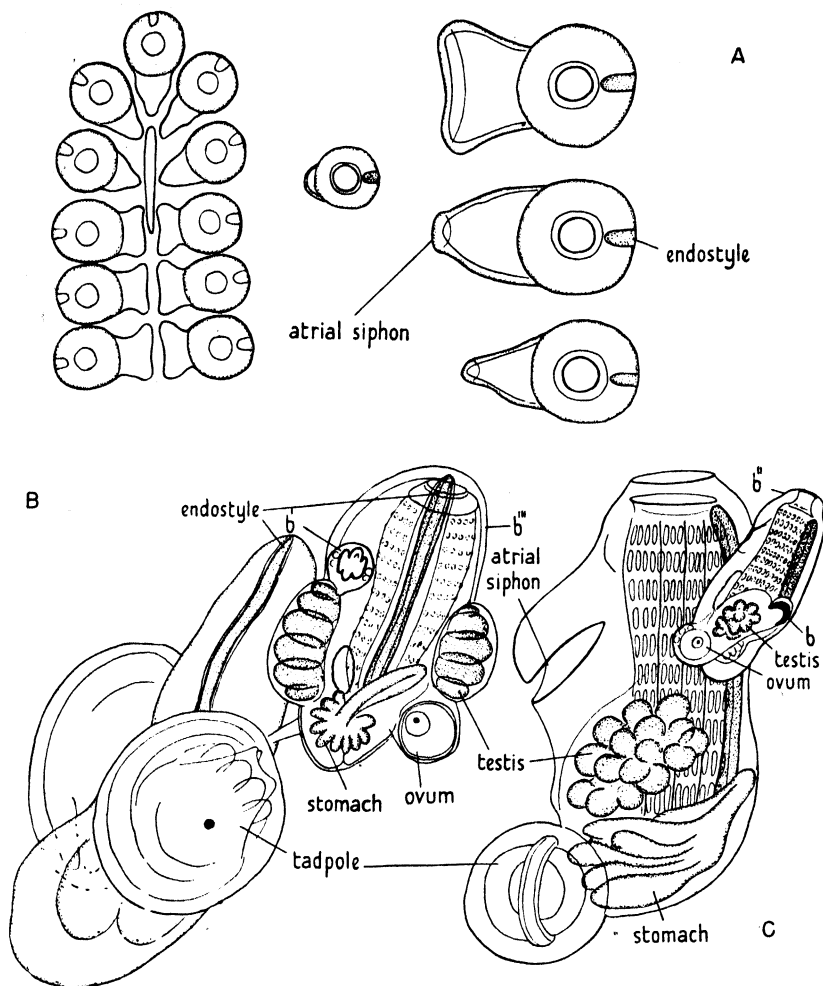
TEXT-FIG. I.

A. Upper surface views of zooids isolated and arranged in a system, showing correlation between size and shape of the atrial siphon and relation to pattern of system, the central figure representing the condition in the first blastozooid generation. B, C. Two zooids from mature colonies showing coexistence and stages of three bud generations in each case. In B the functional zooid is atrophying and about to liberate fully developed tadpoles by rupture, its bud (*b'''*) has perforating stigmata, has a testis on its left side and testis plus ovary on the right, and carries a bud (*b'*) of the next generation on its right side (cp. text-fig. 2c). c shows functional zooid with testis and young tadpole within brood pouch, bud (*b"*) of next generation on its left side with non-perforate stigmata and testis and ovary, together with its own bud (*b*) in late disk stage.

The whole cycle is constant in time for any given temperature, and every stage in the cycle is correlated with an equally well-defined stage of the preceding and, in later stages, of the succeeding generation. As in *Botryllus*, small buds and large buds develop at approximately the same rate and endure as functional zooids for the same period, this last being 8 or 9 days at a temperature of 16°–17° C.

BUD DEVELOPMENT

Buds arise from the atrial wall on either side, about midway along the antero-posterior axis and somewhat toward the ventral side. They appear at a precise stage in the development of the parent zooid, when stigmata rows are represented by imperforate bands of tissue, and arise first as disks of thickened atrial epithelium immediately anterior to the testes. When first discernible the bud disk is three or four cells in diameter, but in the case of those destined to become sexually mature zooids, the disk grows as such until there are about twelve cells in optical section. Then the disk, together with a corresponding area of overlying epidermis, arches progressively until the two layers form an inner closed sphere of atrial epithelial cells and an outer epidermal sphere connected with the parent epidermis by a narrow stalk. At this simplest of morphological stages, two features suggest that the simplicity of pattern is more apparent than real, for an ovum, if one is to be produced at all, is already segregated from the wall of each side of the inner vesicle, while the distal region of the epidermal vesicle shows a slight bulge foreshadowing the ampulla which eventually attaches the bud to the colonial vascular system. This last feature may, of course, be merely a response to the local impinging of the blood-stream circulating between the two vesicles, inducing a local epidermal outgrowth (fig. 2 A, B).

As the bud grows in size, the inner vesicle, when two to three times its original diameter, exhibits all the primary morphological divisions in the form of infoldings and outpushings. A pair of lateral folds, commencing anteriorly, divide the primary vesicle into a central pharyngeal chamber and two lateral atrial chambers. Dorsally a median evagination forms the neural complex, while posteriorly two evaginations form the rudiments of the heart and the digestive tube. At the same time the epidermal ampulla foreshadowed earlier has grown and fused with the nearest adjacent vessel of the colonial vascular system (fig. 2 C).

This stage grows without marked change other than differentiation of the established units until it is several times as large. The heart becomes cut off as a closed vesicle, the intestinal evagination lengthens to form the gut loop, and the midventral band of the pharynx differentiates as endostyle.

The next critical stage is that in which the rows of stigmata are indicated by imperforate transverse bands and the buds of the next generation appear as atrial disks. The new buds follow the course just described, the parent bud continuing to grow until several times as large, to attain functional differentiation with little further morphological change (fig. 2 E).

INITIAL BUD SIZE AND DEVELOPMENT

The relationship between the initial size of buds as represented by the maximum disk stage and the size of subsequent stages including the final one
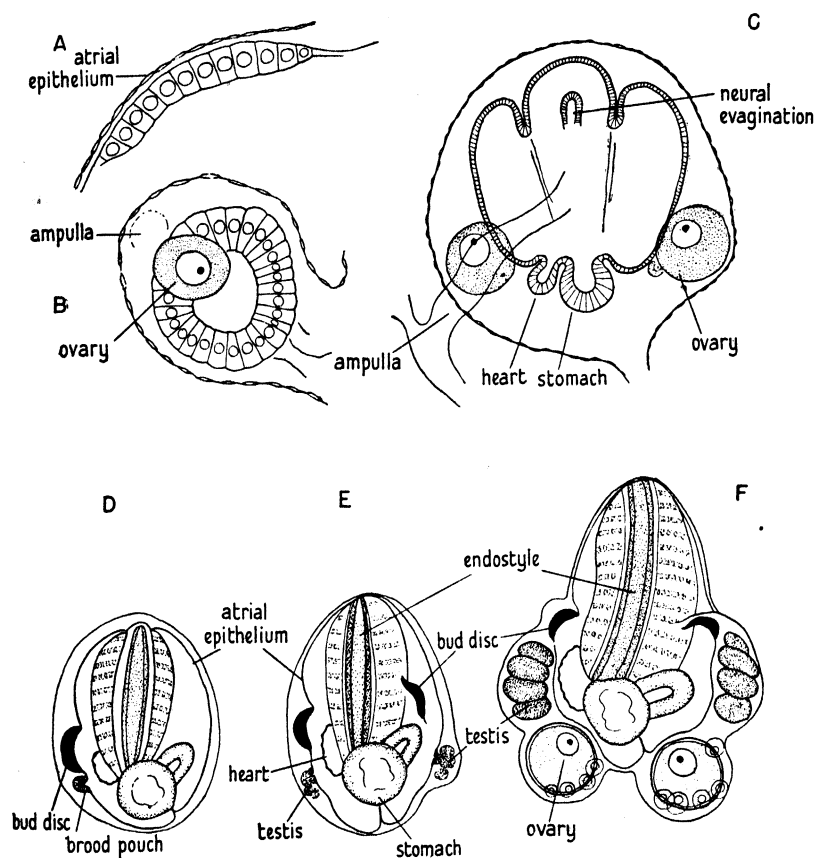
TEXT-FIG. 2. Development of bud.

A. Bud disk at maximum stage showing epidermal and atrial layers. B. Bud sphere with ovum already segregated from wall of inner layer. C. Young bud with ova segregated on each side, ampullary outgrowth attached to colonial vascular system, and inner vesicle folding to form pharyngeal and atrial divisions and neural, cardiac, and intestinal outgrowths. D, E, and F. Three later buds of different size but identical stage showing correlation of absolute size with presence of one or two buds, and variation in gonads.

is direct, and similar to that reported for *Botryllus* (Berrill, 1940). Three sizes of equivalent stages from a single colony and belonging to the same bud generation are shown. All possess maximal bud disks of the new generation, and stigmata are recognizable but not perforate. The bud developing from the smallest disk has its own bud disk only on its right side and has no trace

of gonads. In the case of the disk of intermediate size, the bud has developed atrial disks and also rudimentary testes on each side, but no ovaries (fig. 2 D, E).

The largest disk has produced a maximal bud of the same stage, with bud disk, testes, and ovary on each side. The testes are destined to become functional and consist of several follicles. The ovary consists of one large ovum, and several rudimentary ova which do not grow and eventually degenerate and disappear (fig. 2 F).

These differences are correlated with the size of the bud during the transformation from the disk to the closed vesicle stage, when the large ova and, immediately following, the parental testis and small ova cells are segregated from the vesicle wall. Limitations of size below certain values prohibit such segregation and lead to the results exhibited in the small-disk buds just described.

DOUBLE BUDS

Double buds frequently are formed during the development of maximal buds in well-nourished colonies, apparently on the right side only. They arise from the division of a single rudiment during the late disk stage, one member of the pair forming a maximal bud (and zooid) similar to the bud on the left side, the other always smaller and rarely producing ova or functional testes. The inequality comes from the division of a rudiment of somewhat pear-shaped area, a large and small circular bud area deriving from the large and small ends respectively. All buds, large or small, of the same generation, complete their development and function simultaneously.

GONADS AND PLACENTATION

There has been considerable discussion concerning both the nature of the ovary and of the placenta, different descriptions and interpretations having been presented by Ärnbäck (1923) and by Garstang and Garstang (1928).

According to Ärnbäck,

'a brood pouch develops on each side of the body to receive the embryos. The brood-pouch is of globular form and is a diverticulum of the peribranchial cavity. The oviduct opens into the brood pouch. The embryos undergo their larval development in these uterus-like pouches, in which also fertilization takes place. The egg passes through the oviduct into the brood pouch; before entering the latter the outer follicle has been thrown off, forming a corpus luteum-like structure that lies in the mesoderm and soon degenerates.'

Garstang and Garstang describe the same thing very differently.

'When the egg is ready for fertilization the outer follicle grows out towards the atrial epithelium as a short thick tube. The duct is probably the homologue of the oviduct of other ascidians, but here it functions simply as a fertilization duct. The eggs are not shed into the atrium. Development takes place *in situ*, that is, the larvae are found between ectodermal and atrial walls, in a blood space. . . . The larvae are surrounded by the inner and outer follicles. . . . When a larva is almost ready for emergence, both follicles and the atrial epithelium lying close against them are
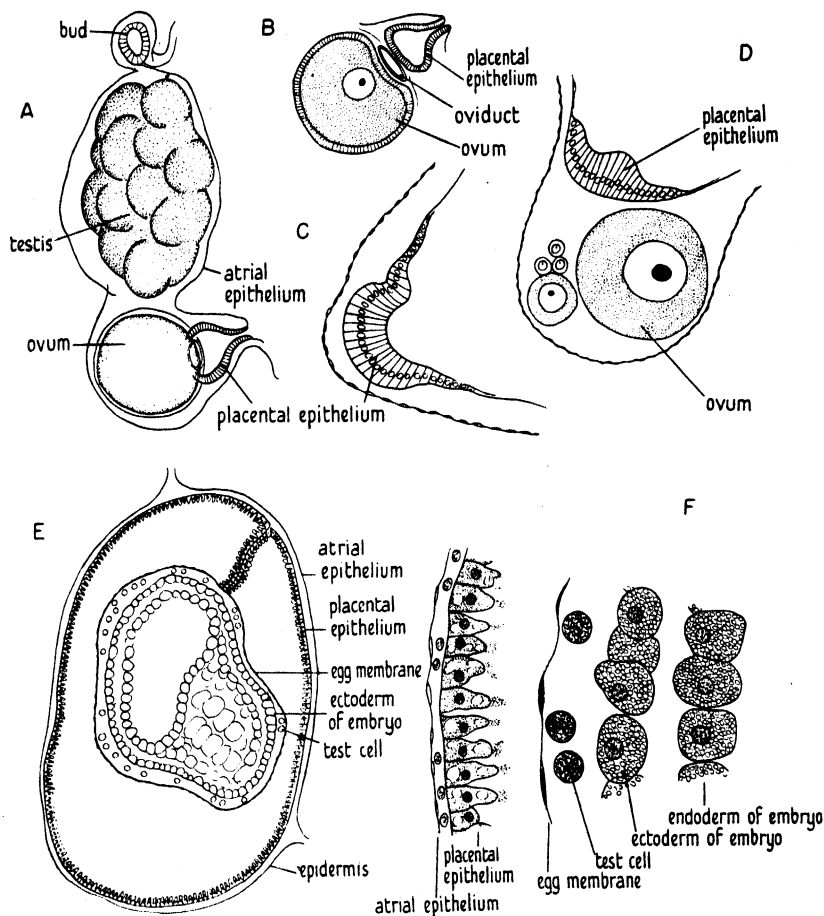
TEXT-FIG. 3. Placental brood pouch.

A. Reproductive complex with anterior bud (at sphere stage), median testis, and posterior ovary consisting of single ripe ovum about to enter brood pouch. B. Developing ovum with germinal vesicle and follicle layer, reduced oviduct, and brood pouch. C. Early stage in development of brood pouch from young bud without sign of gonad. D. Similar stage of brood pouch adjacent to young oocytes. E. Section through functional brood pouch and contained embryo. F. Higher magnification of layers of brood pouch and embryo.

broken through and the larva slips into the atrium. Botrylloides has remarkably little yolk in its eggs, but throughout the embryonic stages, up to the time when the larva frees itself from the follicle, a small tube is invariably present, which connects the outer with the inner follicle. This tube is formed of cubical cells, with clear rounded ends projecting towards the lumen—very like the cells of the outer follicle. At its outer end the tube opens into the bloodspace surrounding the larva, and as this is continuous with the visceral blood-space of the parent, something like a placental communication is established, though the exact way in which nourishment is transmitted is obscure. In Botryllus there is no such tube, and no need of it, because the eggs are provided with a good deal of yolk.'

Commenting on Ärnbäck's account, they suggest that the Plymouth variety of *Botrylloides* is more specialized for vascular nutrition than the Swedish variety described by Ärnbäck.

Both interpretations suffer from being based on insufficient material. All structures described by Ärnbäck and Garstang and Garstang have been identified in Plymouth material, but the account given below confirms and goes beyond that of Ärnbäck.

Shortly before the egg matures it is seen to possess both inner and outer follicle layers, with the germinal vesicle on the side nearest the atrium, and a short oviduct adjacent to that side. This condition is very similar to that of *Botryllus* at the same stage. In *Botryllus*, somewhat later, the egg emerges through the oviduct into the atrium but remains attached by a cup-shaped fold of the atrial wall at the site of emergence, at least until the completion of gastrulation. In *Botrylloides* a flask-shaped invagination of thickened atrial epithelium comes into contact with the oviduct and ovum before maturation, with the passage to the atrium narrowly constricted. This is in conformity with Ärnbäck's description (fig. 3 A, B).

Subsequently the ovum, immediately following the attainment of functional activity by the developing zooid as a whole, ruptures the outer follicle, enters the oviduct and then the atrial involution. This also confirms Ärnbäck, although the discarded outer follicle has not been seen.

These events occupy a very short while, probably not more than 2 or 3 hours. This is a relatively brief part of the whole cycle, and Garstang and Garstang apparently missed the critical period. All the zooids in any one colony are at the same stage and progress in unison through the cycle.

What actually occurs is that the ovum, upon rupturing the follicle, matures and becomes fertilized as it reaches the atrial brood pouch. It retains its inner follicle with basal chorion, together with the so-called test cells in the perivitelline space. Fluid accumulates between the embryo (with its membrane or chorion) and the lining of the brood pouch so that the pouch becomes distended. As it swells it protrudes both out from the body-wall of the zooid and into the atrial cavity, and in so doing the neck of the pouch which originally communicated with the atrial cavity becomes compressed, with its lumen obliterated and the atrial connexion lost. The inner end of the neck remains close to the egg membrane, so that it becomes virtually invaginated

in the expanding pouch. It has almost but not entirely the appearance of a duct and is the placental tube of Garstang and Garstang. Accordingly the structure is a relic of no further functional significance and there is left the problem of the origin and nature of the pouch proper (fig. 3 E).

The pouch arises in the beginning as a thickening of atrial epithelium during the early development of the bud, at about the same time as the appearance of the stigmata anlagen and the bud disks of the next generation. The thickening, which represents local growth, appears adjacent to the ovary, but as far as can be determined it develops at least to some degree even when no ovary is present, and accordingly is not a dependent formation (fig. 3 C, D).

The columnar nature of the epithelium is retained throughout its existence into the functional state. In a brood pouch containing a developing embryo the lining cells are high columnar, vacuolated, rich in secretory granules, and appear to have plasmodial ends. They clearly secrete a coaguable substance into the interior of the pouch. Throughout the period of development of the egg the fluid increases and the pouch becomes more and more distended (fig. 3 E, F).

EMBRYONIC DEVELOPMENT

Basically the development of the egg of *Botrylloides* is like that of *Botryllus* up to and including the tadpole stage, with two obvious differences. These are the relatively small size of the *Botrylloides* egg, and the relatively large and precociously formed space formed within the archenteron and between the endoderm and ectoderm. These two features appear to be associated with the extra-embryonic nutrition, the pressure of the external fluid invading the embryo and in a sense producing an oedema between the layers, and the external nutrition compensating for reduction in egg size to produce the same end result. Actual nourishment of the developing embryo by the secretory lining of the brood pouch is, of course, not proven, and further investigation along physiological lines is desirable. There is, however, an active secretion towards the embryo, and there is the fact that the smaller egg, which is also the less yolky, develops as far as that of *Botryllus*.

The egg cleaves in typical unmodified ascidian manner, the eight-cell stage consisting of two tiers of four cells arranged in bilateral pattern as described by Conklin (1905) for *Styela*. Gastrulation takes place between the sixth and seventh cleavage. Subsequent development up to the tadpole stage, apart from the extensive intertissue spaces, is similar to that of *Botryllus* and has been adequately described by Garstang and Garstang (1928). These authors, however, describe hatching as a breaking through of the embryo into the atrium, but there seems to be little evidence of this. In fact the tadpole is relatively so large that it would in any case be a matter of great difficulty to escape in that way, and also the parent zooids are usually commencing regression at the time. Rupture of the external wall of the brood pouch liberating the tadpole directly into the common cloacal cavity appears to be the usual method employed (fig. 4 A–G).
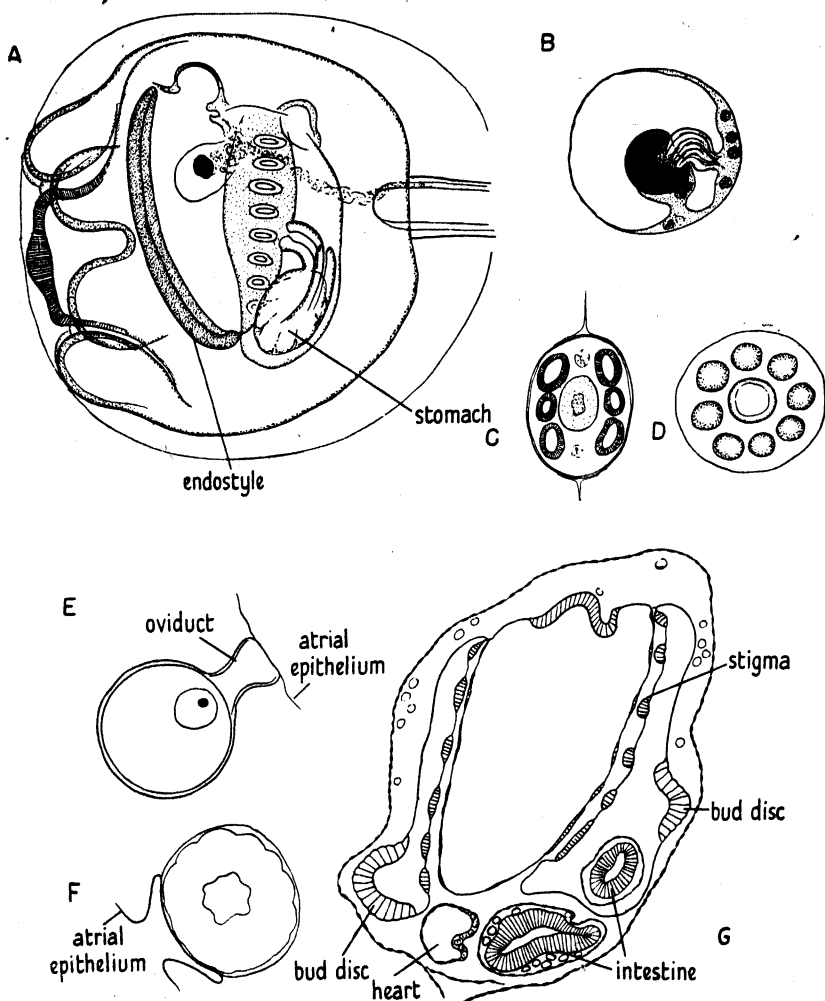
TEXT-FIG. 5.

A. Lateral view of trunk of tadpole of *Botrylloides* showing preoral lobe ringed by epidermal ampullae, sensory vesicle with nerve-strand passing to dorsal side of tail, protostigmata, and digestive canal. B. Enlarged sensory vesicle showing single-celled otolith penetrated by light sensitive neurons to form photolith. C. Cross-section of tail showing 3 muscle-cells on each side. D. Anterior view of tadpole showing central lobe and ring of 8 ampullae. E. Ovum and oviduct of *Botryllus* for comparison. F. Gastrula of *Botryllus* supported by fold of atrial lining. G. Vertical section through young bud of *Botrylloides* showing atrial bud disks, heart, digestive canal, and pharyngeal and atrial components of developing gill slits (stigmata).

developing from large eggs and is in contrast to the 90° rotation typical of all large-egged entergonids, e.g. *Amaroucium*, *Perophora*, &c. The avoidance of rotation, as in related forms, is due to the oblique curving of the tail around the developing trunk so that torsion is not applied to the congested tail fin (fig. 4 F).

The sense organ is a 'photolith', an organ first described for *Botryllus* by Caswell Grave (1924) and so named by Garstang and Garstang (1928). It arises in the late development of the tadpole as a typical unicellular otolith. Later still, in fact almost belatedly, several adjacent neurosensory cells from the wall of the sensory vesicle grow in towards it and penetrate deeply into the mass of pigment. As in *Botryllus*, the tadpoles are both light- and gravity-sensitive in consequence (fig. 5 A, B).

Within the trunk the essential ascidian structure is well established, including endostyle, closed siphons, a vertical row of well-perforated protostigmata on each side, and a more or less differentiated digestive tube. Anteriorly there is a relatively wide and short stalk, the pre-oral lobe of Willey (1894), on the distal surface of which the three simple adhesive organs are formed. There is some doubt whether these organs are active in their normal function of attaching the tadpole when it first settles. The central stalk with its adhesive organs is surrounded by a ring of eight epidermal ampullae that in any case form the permanent organ of attachment, and the ampullae appear more able to assume this function at the time of settling of the tadpole than at the comparable time in *Botryllus* (fig. 5 D).

The active free-swimming period is on an average about 1 hour, with approximately 10 per cent. of the tadpoles remaining active as long as 12 hours. One day after the settling the siphons are prominent and the first blastozooid is visible as a small vesicle. At the end of the second day, the heart is beating, the siphons contractile, and the cilia of the gill slits just becoming active. As seen from the right side there are eight long protostigmata extending across the width of the branchial sac. The eight ampullae more or less radiate from beneath the base of the endostyle over the substratum.

The oozooid survives in an active condition for about a week, when it resorbs as the first blastozooid grows into functional activity. There is usually but one blastozooid of this generation, that of the right side, the one on the left usually starting to develop during metamorphosis but remaining abortive and disappearing. The ampullae of the oozooid survive the resorption process and link up with the ampullary vascular system of vessels established during succeeding blastozooid generations. The ampullae are independently contractile and aid in maintaining the colonial circulation (cp. Bancroft, 1899).

The first blastozooid differs from the oozoid mainly in having four rows of definitive stigmata on each side in place of the eight or nine undivided protostigmata. It is also without trace of gonads and has an atrial siphon similar to that of the oozooid. Subsequent blastozooid generations, when well nourished, produce both right and left buds, increase in number each generation, and increase in individual size as well; with the successive increases in

individual size, the number of rows of stigmata increases from four to a dozen or more, the atrial siphons become first conical and then flute-mouthed, and orientate to establish star or ladder systems, while gonads develop, first male only, then later hermaphrodite (fig. 2 D–F, 4 H–I).

DISCUSSION

Botrylloides may be regarded as an evolutionary specialization of *Botryllus*. Whether this is true or not, the two genera are closely related and they can be compared in a strictly relative manner. Obviously, since the respective adult structure of zooid and colony is the product of certain basic developmental processes in each case, the differences exhibited by the two genera may be correlated with such differences in developmental activities.

In the case of bud development, the buds in both genera arise in the same relative positions at the same critical developmental stage of the parent bud, and involve the same tissues. There is the same general co-ordination of development of branchiae and gonad with initial bud size. In *Botrylloides*, however, there is a minor but significant difference in that the bud rudiment, in becoming a closed vesicle and thereafter, becomes relatively more elongated along its antero-posterior axis and relatively smaller in diameter. Three features appear to be correlated more or less closely with this growth difference. In conformity with the elongation of the developing branchial sac, there is space for more rows of stigmata to be laid down at the critical stage. The relative narrowness of the cylindrical form of the bud and zooid restricts somewhat the area from which bud disks develop, so that the maximum areas attained are relatively smaller than those finally produced in *Botryllus*. Consequently, mature *Botrylloides* zooids in their maximum state have one mature ovum and several small degenerate ova, comparable to the mature *Botryllus* zooid at its minimum condition (cp. Berrill, 1940), the maximum state in this form possessing four or five mature ova on each side. That is, the difference in the gonads of the two genera in a quantitative sense is a function of absolute size of buds at their inception. Thirdly, the difference in pattern of systems in the colony is due to differences in the shape of the atrial siphons of the zooids. That of *Botrylloides* appears to be the result of continued growth beyond the *Botryllus* condition involving a relative increase in growth rate of the transverse axis. This may in turn be related to the more basic differences in growth rates of the longitudinal and transverse axes of the developing buds taken as a whole.

Other intergeneric differences come under another category. The shift of the ovary from a position lateral to the testis, as in *Botryllus*, to one posterior to it in *Botrylloides* is apparently a difference in primary pattern not related to differences in dimensions or cell numbers, although in a more obscure way it may yet be an elongation effect. The development of a brood pouch as a local thickening of atrial epithelium at an early stage in bud development implies a local reactiveness and cellular differentiation absent in *Botryllus* and can only be an addition to the repertoire of cell specializations. Differences in

fertilization procedure, if any, in early development of the egg, nature of embryo, its location and nutrition, and finally method of liberation may well be only consequences of the presence and nature of the primary brood-pouch epithelium as a local specialization of the atrial wall.

The suggestion is accordingly made that the various minor and major differences between the genera *Botrylloides* and *Botryllus* are the consequences of two basic differences, in the relative growth of the two primary axes of the bud, and a new cell specialization.

SUMMARY

The life-cycle of *Botrylloides* is described in comparison with that of *Botryllus* and, as far as possible, as a space-time continuum.

The origin, growth in size, form changes, and time relationships of the egg, embryo, and bud are described. Bud-development follows the same direct course as in *Botryllus*, but varying in size at each respective stage with the size of the initial bud disk. The gonad may be absent, male with or without rudimentary ova, or functional hermaphrodite according to the size of the bud disk and of the critical stage at which the various components of the gonad are segregated. Buds of each generation arise at the same precise stage of development of the parental buds. As each bud generation completes its development, the whole of the preceding generation is resorbed, and the newly matured and fertilized eggs of the new generation commence their development. The duration of egg development up to the formation of the active tadpole lasts until a few hours before the resorption of the parent zooid and escape to the common cloacal cavities is made directly by rupture of the parental body-wall. Eggs do not develop in the atrial chamber as in *Botryllus* but become enclosed in a special brood pouch, a unique structure arising early in the development of the bud and apparently independently of the presence of the gonads. Some form of extra-embryonic nutrition is evident and is supplied by the secretory lining of the placental brood pouch.

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Some Observations upon the Golgi Elements of the Anterior Pituitary Cells of Normal and Stilboestrol-treated Male Rats, using the Sudan Black Technique

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With one Text-figure and one Plate

INTRODUCTION

THIS investigation was undertaken in order to discover whether the Golgi elements of anterior pituitary cells possessed the duplex structure described by Baker (1944) for cells of certain vertebrates and invertebrates, after fixation in formal-calcium chloride and staining in sudan black.

Pituitaries of normal mature male rats and comparable animals implanted with tablets of stilboestrol were used. The latter were studied for comparative purposes, since it is known that both synthetic and natural oestrogens activate the glandular cells of the anterior hypophysis, causing, among other changes, hypertrophy of the Golgi elements (Severinghaus, 1937; Foster, 1942).

METHODS

Six control and nine experimental rats with an average weight of about 150 gm. were used. The animals were killed by a blow on the neck, the experimental animals being killed at varying times after the subcutaneous implantation of a 10 mgm. tablet of stilboestrol.

The technique for demonstrating the lipoids of the Golgi zone with sudan black followed that described by Baker (1944). Since, in some instances, there was a tendency for a precipitation of the dye on the surface of the frozen sections, the following method was devised to overcome this difficulty.

The sections were first washed to remove the formal-calcium-cadmium chloride solution in which they had been stored and were then dipped in a 12 per cent. solution of gelatine, kept liquid in the 37° C. incubator. The slide was then lifted out and allowed to drain for half a minute, when the gelatine on the back was wiped off. The thinnish layer of gelatine on the section side of the slide was allowed to dry off until it became tacky, and was then coagulated by holding the slide, gelatine face downwards, beneath the surface of a dish of 90 per cent. alcohol. The section was then transferred to the filtered sudan black solution. The time necessary for adequate staining was, of course, found to be rather longer by this method and varied with the

thickness of the gelatine film. In practice, the slide was periodically removed from the dye, rinsed in 50 per cent. alcohol, and examined under the microscope.

At the completion of staining, the slide was rinsed in 50 per cent. alcohol and the gelatine film was readily removed by immersion in warm water. After further washing in distilled water, the preparation was mounted in glychrogel (Cowdry, 1943).

<div align="center">RESULTS</div>

1. *Normal Animals*

(a) *The Position of the Golgi Zone.* In many instances the Golgi zone was clearly identifiable in the glandular cells of the pars distalis and details of its structure could be made out. This was particularly true of the basiphil cells, Golgi elements of which, as is well known, are revealed in Nassonov–Kolatchev preparations as fairly large spheroidal nets, generally situated at a distance from the nuclear membrane (cf. Text-fig. 1, *i* and Pl. 1, *i, iv, v*; Text-fig. 1, *ii–vi*). The details of the Golgi zones of the acidophil cells were, generally speaking, not so clearly defined, but the position and shape of the zone were almost exactly similar to those found in Nassonov–Kolatchev preparations; the zone was like a peruque capping the nuclear membrane. (Cf. Text-fig. 1, *i*, and Pl. 1, *iii, vii, viii*; Text-fig. 1, *ii, iii, iv*.) The Golgi elements in the small chromophobes were more difficult to make out, but in a number of instances it was possible to see that in some it had the shape and position characteristic of the acidophil type (Text-fig. 1, *iii*), while in others, its shape and position were such as are to be found in the basiphil cell (Pl. 1, *vi*). These observations were in agreement with those of Severinghaus (1936), who observed that in the mature gland, the two distinctive types of Golgi element characteristic of the acidophil and basiphil cells were present also in the chromophobes. Finally, the large chromophobes showed the same two forms of Golgi zone as was noted in the chromophils and small chromophobes —they are in fact, as has been shown by Severinghaus (1933), chromophils in the process of granule depletion.

Although the sections were not counterstained, very little difficulty was encountered in the identification of the cells types mentioned above, particularly as the preparations were studied in conjunction with stained Nassonov–Kolatchev material. The small chromophobes were the smallest elements of the cell population and were devoid of granules. The yellowish refractile granules of the acidophils were easily recognizable, and the basiphils were recognizable by means of their larger size, their indistinct granules, and characteristically placed Golgi bodies. The large chromophobes of the acidophil type were either partially or completely devoid of granules, as were those of the basiphil type. In the latter, this was reflected in a considerably reduced intensity of cytoplasmic staining with the sudan black.

(b) *The Structure of the Golgi Zone.* The Golgi zones of both acidophil and basiphil cells possessed three features in common and, in fact, differed
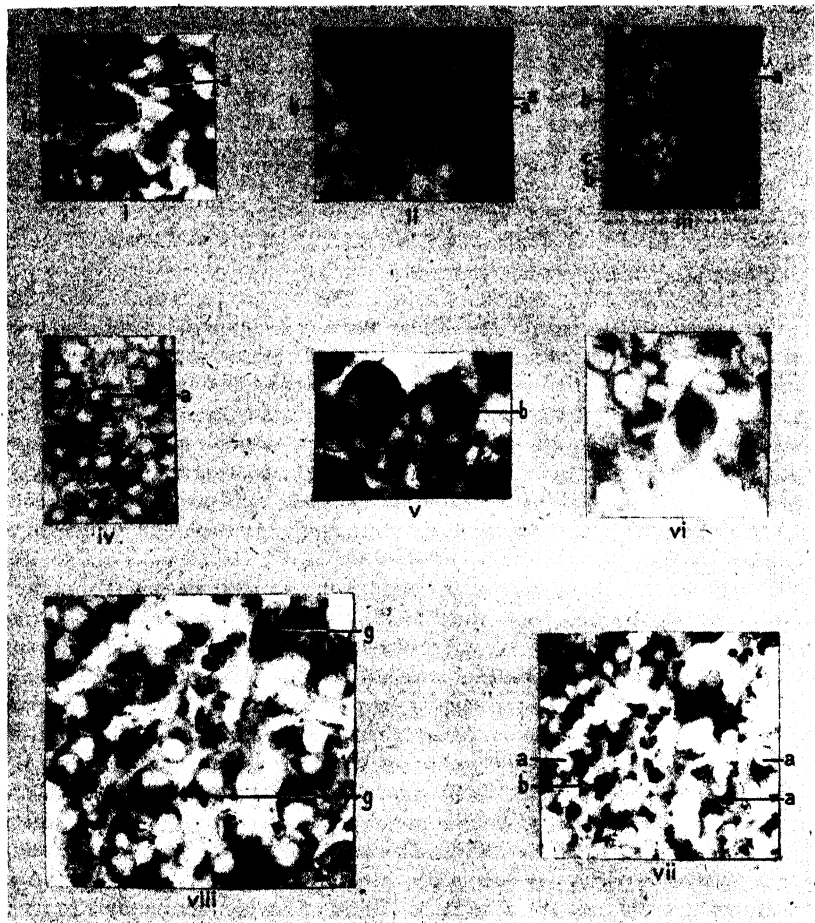
TEXT-FIG. I.

i. Normal ♀. A, peruque-like Golgi element capping the nucleus of an acidophil cell; B, spheroidal Golgi zone of a basiphil cell. Nassonov–Kolatchev. Modified Mallory stain. ×650.

ii. Control ♂. A, Golgi zone of acidophil cell; B, Golgi element of basiphil cell. ×325.

iii. Control ♂. A, acidophil cell with Golgi zone immediately above and capping the nucleus; B, basiphil cells with characteristic Golgi elements, in which vacuoles are faintly discernible; C, acidophil type of small chromophobe with small Golgi zone in contact with lower edge of nucleus. ×325.

iv. Control ♂. A, acidophil cell with Golgi element consisting of granules of dense lipoid. ×325.

v. Control ♂. B, one of two large basiphil cells showing general form of Golgi zones, and their relation to nuclei. ×650.

vi. Control ♂. Curiously shaped basiphil cell in which vacuoles are faintly detectable in the Golgi zone, the central region of which is occupied by diffuse lipoid. ×650.

vii. Experimental ♂. Stilboestrol 32 days. A, acidophils with hypertrophied Golgi zones in which the diffuse lipoid and dense lipoid material is readily seen; B, hypertrophic Golgi area of a basiphil showing similar features to the above. ×325.

viii. Experimental ♂. As above. G, Golgi elements showing rows of enlarged vacuoles enveloped in dense lipoid material. This is a more highly magnified area from the centre of *vii.* ×650.

from one another only in the two respects already mentioned—(*a*) in shape, and (*b*) position in relation to the nucleus.

The three features were:

1. A zone of diffuse, less deeply staining sudanophil material, embedded in which were:

2. Small clear vacuoles, and

3. Strongly sudanophil elements.

The position and shape of the diffuse sudanophil material were primarily responsible for determining the morphology of the Golgi zone as a whole. That is to say, the difference between the acidophil and basiphil types was directly related to differences in the size, shape, and position of the diffuse sudanophil material and not to the nature of the vacuoles and the strongly sudanophil inclusions (cf. Pl. 1, *i* and *vii*). In some cases, particularly in small cells, where the diffuse sudanophil material could not be readily made out, the distribution of the strongly sudanophil material was sufficient to characterize the type of Golgi zone (Pl. 1, *iii*).

The vacuoles, although normally small, were variable in size (Pl. 1, *viii*), and, in occasional instances, were to be seen outside the zone of diffuse sudanophil material (Pl. 1, *iv*). The relationship between these vacuoles and the strongly sudanophil elements was not always clear-cut. Sometimes, as in Pl. 1, *iv*, *v*, *viii*, some of the vacuoles appeared to be unconnected with the sudanophil elements, but this may perhaps have been due to the extreme thinness of any sudanophil film investing them.

In those instances where there was clearly a relationship between the intensely sudanophil particles and the clear vacuoles, the former appeared either as small granules of which one and sometimes two were seen to be in contact with the edge of a vacuole (Pl. 1, *iv*, *v*, *vii*) or else as sudanophil arcs capping single vacuoles (Pl. 1, *ii*, *viii*). There was evidence in some instances that these 'caps' were approximately crescentic in form (Pl. 1, *viii*), but in others they appeared to be of a uniform thickness (Pl. 1, *iv*, *v*). A few examples of vacuoles apparently completely invested with a sudanophil film were observed (Pl. 1, *viii*).

In some cells there was a high proportion of small, strongly sudanophil, granule-like bodies of variable size, apparently unrelated to any external or internal vacuole. Pl. 1, *iii* shows a rather small acidophil type cell whose Golgi zone consisted entirely of these apparently solid granules.

Thus, in these Golgi bodies, in addition to the region of diffuse sudanophil material usually present, there were observed to be:

1. Vacuoles apparently unrelated to the strongly sudanophil bodies;

2. Strongly sudanophil elements apparently unrelated to vacuoles; and

3. Vacuoles associated with caps or granules of strongly sudanophil material.

2. *Experimental Animals*

(*a*) *The Position of the Golgi Zone.* Any alteration in the position of the Golgi zone was clearly due to the generalized hypertrophy of the whole area. This hypertrophy in response to oestrogens is already well known from observations based upon the study of material prepared by the Nassonov–Kolatchev method (Severinghaus, 1937). When the hypertrophy was considerable, there was a tendency for the Golgi region of the basiphil cells to encroach upon the nuclear region and make contact with the nuclear membrane (Pl. 1, *xi*), and this resulted in a somewhat acidophil-like morphology (cf. Pl. 1, *xi* and *viii*). In neither type of cell, however, was there evidence of any fundamental shift in position, and only occasionally was difficulty encountered in deciding the type of cell under observation.

(*b*) *The Structure of the Golgi Zone.* The three features mentioned as being characteristic of the Golgi regions of normal cells were again readily seen in the experimental animals, and, generally speaking, the differences between the two were quantitative rather than qualitative, although certain qualitative differences were observed.

Even after a relatively short period of implantation with stilboestrol there was quite a marked increase in the amount of diffuse sudanophil material, relative to the cytoplasmic volume (cf. Pl. 1, *v* and *xi*; *vi* and *x*), and with more prolonged treatment the increase was unmistakable (cf. Pl. 1, *viii* and *xiii*; Text-fig. 1, *ii* and *vii*). Concurrently, the intensity of the staining of the diffuse material also increased, but this may perhaps have been due to a closer packing of the sudanophil particles and hence may have been a quantitative and not a qualitative change. The Golgi elements of all cell types were similarly affected, but since one one of the characteristic effects of prolonged treatment with oestrogens is a granule depletion in the chromophil cell (Severinghaus, 1937), the cells observed in those glands subjected to a more prolonged treatment were commonly extremely degranulated—they were in fact large chromophobes.

The effect of the oestrogen upon the vacuoles and strongly sudanophil elements was, after fairly prolonged treatment, quite clear-cut. First, there was an increase in both the amount of vacuolation (cf. Pl. 1, *vii* and *xii*) and the amount of strongly sudanophil material (cf. Pl. 1, *v* and *xi*), although the latter was not always so marked. As in the controls, there were vacuoles apparently unrelated to sudanophil elements and there were granular sudanophil elements unassociated with any external or resolvable internal vacuole (Pl. 1, *ix, xii, xiv*). Secondly, there was a qualitative change resulting from the coalescences of vacuoles. This, in its extreme form, is shown in Pl. 1, *xv* and Text-fig. 1, *viii*, where vacuolar chains of large size were produced; in other instances, the evidence for vacuole fusion was based on the occurrence of vacuoles considerably above the normal size (Pl. 1, *xiii, xv*). These were often associated with several sudanophil granules and crescents, instead of the more usual one or two seen in the controls. The sudanophil elements were generally of the same type as in the controls, that is to say: crescents (Pl. 1, *ix*,

x, *xi*), caps of apparently uniform thickness (Pl. 1, *xi*) and occasionally continuous films (Pl. 1, *ix*). Appearances such as those shown in Pl. 1, *xiv*, where the sudanophil material was elliptical in shape, suggested that ring-like configurations were also present.

The results described above show that the essential structural features of the Golgi body were maintained after fairly prolonged treatment with stilboestrol. The hypertrophy produced by oestrogens was due to (1) an increase in the amount of sudanophil material of both types, and (2) an increase in the amount of vacuolation.

DISCUSSION

Since sudan black is known to have a high and apparently specific solubility in lipoids (Baker, 1944) it can be justifiably assumed that the sudanophil material of the Golgi elements just described consists, at all events in part, of lipoid substances. The Golgi zones of the cells of normal and experimental animals thus consist of regions containing diffuse lipoids in which are embedded three sorts of structures: (*a*) clear vacuoles, (*b*) lipoid-containing granules, and (*c*) clear vacuoles partially or completely invested with dense lipoid material. It is reasonable to believe, for reasons indicated by Baker (1944), that the technique preserves these structures in a form not greatly dissimilar to that obtaining in the living cell and, in any case, it seems improbable that any distortion produced would exceed that caused, for example, by the Nassonov–Kolatchev procedure. There is in fact a very close similarity between the morphology of the Golgi zone as revealed by the latter method, and that demonstrable by the use of sudan black.

Severinghaus (1932), using the Nassonov technique, showed that two sorts of Golgi element, associated respectively with acidophils and basiphils, were present in the anterior lobe of the rat pituitary. The result of the present investigation supports his observations. The fact that two distinctive forms of Golgi element regularly exist in this way in the two sorts of chromophil cell (Severinghaus, 1933, 1937) of the rat, the guinea-pig (Kirkman, 1937), and perhaps less markedly in the cat (Dawson, 1946) is of theoretical interest, since it suggests that the Golgi zone, in spite of its lability, must be linked to the 'cytoskeleton'. In the rat, the characteristic Golgi zone morphology of the two types of chromophil cell is retained after treatment with oestrogens, except where the dosage is particularly heavy; but the evidence again suggests that the pattern of the Golgi zone is related to the ultrastructure of the cytoplasm.

The Golgi material of the anterior pituitary cells of the rat, as revealed by the Nassonov–Kolatchev technique, for example, consists of a network of osmiophil threads and granules associated, according to observers such as Severinghaus (1937) and Ayers (1941), with clear vesicles or vacuoles. The latter described such vesicles as having three different relations within the cell: (1) the smallest, appearing as 'clear, tiny ovoid spaces incorporated in the strands of the Golgi net', (2) '. . . slightly larger vesicles eccentrically

located so that only a thin rim covers the outer side'—such vesicles being up to three times the size of the previous type—and (3) vesicles present in the cytoplasm, some of which 'have a bit of osmiophil material adherent'. There can be little doubt that these vesicles are similar to the vacuoles observed in the sudan black preparations, and it seems likely that the stranded osmiophil material in which they are embedded, or with which they are associated, corresponds partly to the diffuse and partly to the denser lipoid already described. Also, cytoplasmic vacuoles associated with dense lipoid material were occasionally observed in the sudan black preparations (Pl. 1, *iv*).

The 'classical' reticulate appearance of the Golgi elements in pituitary cells as seen after the Nassonov and similar techniques may well be due to the shrinkage and distortion of the complex revealed by the sudan black method. Some evidence in support of this notion has been brought forward by Worley (1943 *a* and *b*, 1944), who showed (1) that structures comparable to the Golgi elements in regard to their position in the cell and their relation to other cytoplasmic parts (e.g. secretion droplets, &c.) were, in various invertebrate and vertebrate cells, stainable supravitally with methylene blue, and (2) that shrinkage induced by dessication in many instances resulted in a picture very similar to that normally produced by the customary osmium tetroxide impregnation techniques. In the living pancreas, for example, there was a progressive distortion of the stained spherical bodies from which, after a time, there extended thread-like processes whose anastomoses with one another produced a network.

Furthermore, the observations of Worley are in support of those of Baker (1944) and the writer, in that they provide evidence for a duplex structure of the Golgi element—a concept very extensively developed by the investigations of Hirsch (1939). Worley showed that the Golgi vesicles in the cells he studied regularly progressed from a small methylene-blue-stained granular stage to one where the granule had enlarged and differentiated into a clear vacuole with a chromophil pellicle of varying thickness. The vacuole was the site of production of the secretion product, liberation of which was associated with the fragmentation of the chromophil cortex. It seems probable that these two parts of the vesicle correspond to Hirsch's 'Internum' and 'Externum', and the writer believes that this correspondence may be extended to the vacuoles and the associated lipoid elements of the Golgi bodies of the rat anterior pituitary. It is possible, moreover, that the dense sudanophil structures unassociated with vacuoles are equivalent to Hirsch's 'Presubstanz', from which the corticated vacuoles of the fully developed Golgi zone are probably derived. Baker (1944) has suggested the extension of the term 'Externum' so as to include the diffuse as well as the dense lipoid material.

The hypertrophic effect of oestrogens upon the Golgi element of anterior pituitary cells has been clearly established (Severinghaus, 1937), but sudan black because of its high solubility in lipoids gives more precise information about the details of this hypertrophy. Severinghaus (loc. cit.) has described an increase in the vacuolation of the Golgi zone after oestrone injections and

the method used in this investigation showed that not only may there be an increase in the number of vacuoles, but, after prolonged treatment with stilboestrol, coalescence of these vacuoles occurred (Pl. 1, *xv*). There appeared also to be an increase in the amount of both diffuse and dense lipoid material and in some instances coalescence of the former to produce sudanophil strands. Such changes as these, considered in relation to changes known to occur in the Golgi bodies of other endocrine as well as exocrine glands (Bowen, 1929; Kirkman and Severinghaus, 1938; Foster, 1942; Bourne, 1942), seem to indicate a state of heightened secretory activity. Finally, as has been suggested before (Severinghaus, 1936; Foster, 1942*a*), it is thought likely that the physiological inhibition produced by oestrogens is due either to over-activation of the secretory mechanism (of which the Golgi element is almost certainly a part) or to an ultimate interference with some stage in the metabolism of the hormonal secretion product.

SUMMARY

1. It was found possible to demonstrate the Golgi zones of the cells of the anterior pituitary (pars distalis) of the rat with sudan black. The position and general morphology of the Golgi bodies closely resembled that revealed by the standard osmium tetroxide techniques.

2. The Golgi zone was generally found to contain three sorts of element: (*a*) diffuse sudanophil material in which were embedded: (*b*) clear vacuoles and (*c*) strongly sudanophil bodies. The latter were often associated with the surfaces of the vacuoles, when they were in the form of (1) crescentic caps, (2) partial or complete rims of apparently uniform thickness, or (3) small granules. Sometimes the strongly sudanophil bodies were granular and unassociated with vacuoles; there were also vacuoles with no demonstrable lipoid material in association with their surfaces.

3. The effect of stilboestrol was to cause hypertrophy of the Golgi zones and this was associated with (*a*) an increase in volume of the region of diffuse lipoids, (*b*) an increase in vacuolation, and (*c*) a rather more variable increase in the amount of strongly sudanophil material. These changes would appear to indicate a state of heightened secretory activity.

4. The strong affinity of the Golgi area for sudan black suggests that it consists, at any rate in part, of lipoids.

EXPLANATION OF PLATE I.

Figs. *i–xv* are from camera lucida drawings.

i. Control ♂. Basiphil cell showing general form of Golgi zone.

ii. Control ♂. Acidophil cell. Upper focal plane, showing diffuse lipoid and vacuoles with caps containing dense lipoid.

iii. Control ♂. Acidophil cell with peruque-like Golgi zone consisting of dense lipoid particles.

iv. Control ♂. Large basiphil cell with vacuoles outside area of diffuse lipoid, and vacuoles unassociated with dense lipoid.

v. Control ♂. Basiphil cell, showing diffuse lipoid, and vacuoles associated with incomplete dense lipoid rims or granules.

vi. Control ♂. Basiphil type of small chromophobe showing Golgi zone.

vii and viii. Control ♂. Acidophil cells showing vacuoles of varying size with dense lipoid rims and crescents, and also isolated granules containing dense lipoid.

ix. Experimental ♂. Stilboestrol 6 days. Acidophil cell, showing vacuoles with complete investments of dense lipoid.

x. Experimental ♂. Stilboestrol 19 days. Acidophil cell showing enlarged area of diffuse lipoid and numerous vacuoles of varying size, with crescents, rims, and granules of dense lipoid.

xi. Experimental ♂. Stilboestrol 19 days. Basiphil cell showing enlarged area of diffuse lipoid, vacuoles of varying size, some unassociated with dense lipoid.

xii. Experimental ♂. Stilboestrol 19 days. Acidophil cell showing vacuoles outside zone of diffuse lipoid.

xiii. Experimental ♂. Stilboestrol 49 days. Acidophil cell showing greatly increased zone of diffuse lipoid.

xiv. Experimental ♂. Stilboestrol 43 days. Acidophil cell showing two markedly elliptical vacuoles with rims of dense lipoid.

xv. Experimental ♂. Stilboestrol 56 days. Basiphil cell showing hypertrophy and partial fusion of the vacuoles and coalescence of the dense lipoid material to form strands.

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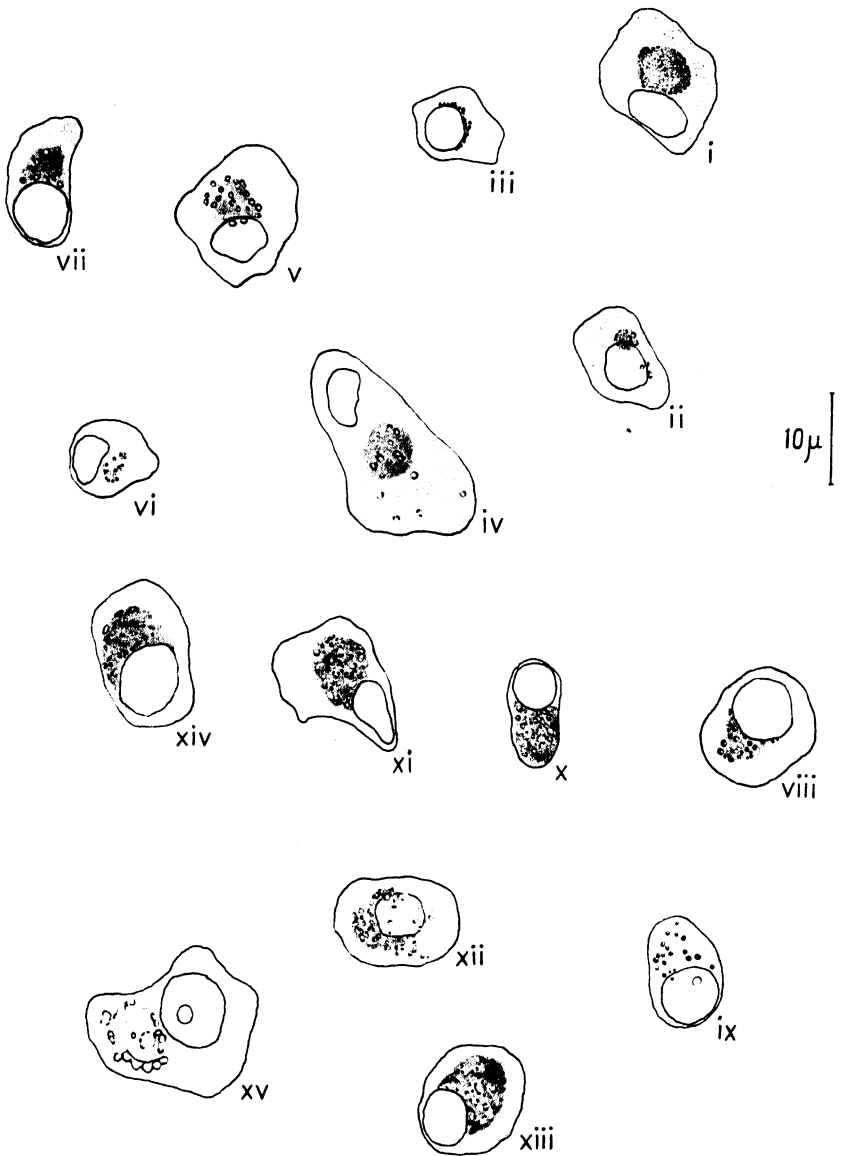
C. L. FOSTER.—PLATE I

A Cytological Study of the Alimentary Tract of the Domestic Fowl (*Gallus domesticus*)

BY

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With two Plates and four Text-figures

CONTENTS

INTRODUCTION

THE work recorded in this paper was undertaken in order to investigate the morphological changes which the cytoplasmic components undergo during different physiological phases induced by fasting and food stimuli, and to study the role of the Golgi material and mitochondria in secretory phenomena. The examination of the proventriculus, gizzard, and intestine yielded information on the cytology of gastric and intestinal epithelia which does not seem to have been previously recorded.

While there are extensive histological studies on the alimentary tract of the fowl (Clara, 1926–7; Calhoun, 1933) there are few published accounts of its cytology. Workers in this field, owing to the suitability of the material, concentrated mainly on cytoplasmic changes during embryonic life. Brief

cytological accounts of the mitochondria and Golgi material of various cells which compose the lining of the intestine of birds are given by Clara (1926-7). Argeseanu and May (1938), in their work on the intestinal epithelium of domestic fowl, outline, as the principal aim of their work, similarities and differences which exist between the cell components at various stages of development. Their observations on the Golgi material are based exclusively on rather unreliable silver nitrate impregnations. Hibbard (1942) describes the changes in form and position of the Golgi material in the proventriculus and gizzard of the domestic fowl throughout the stages of embryonic development. None of the papers quoted contains a complete survey of all the cytoplasmic structures in any part of the alimentary tract investigated by the present writer.

There are numerous papers dealing with the cellular changes which take place during digestion and absorption; these include a wide variety of material ranging from the simplest protozoa to the complicated intestinal epithelium of mammals. Some of these works will be discussed in a later part of this paper.

MATERIAL AND METHODS

The material used in the present work consisted of samples taken from different parts of the intestinal tract, namely, proventriculus, gizzard, duodenum, ileum, caeca, and rectum of the domestic fowl (*Gallus domesticus*).

Material was obtained from young chickens (Brown Leghorn) aged between 3 weeks and 3 months old. Chickens, battery reared, of uniform breed and management, and free from disease, were chosen. All specimens were killed and small pieces of the tissues immediately dissected out and placed in one of the fixing fluids.

At least two birds were used to investigate each physiological phase. When the secretory cells were found to be active, and marked morphological changes observed, additional specimens were used as a check to avoid mistakes due to technical errors. At first, birds were chosen after 24 hours fasting with free access to water. Observations on all subsequent phases were undertaken at various times after feeding. Each feeding experiment was preceded by 24 hours fast, after which food was given freely. Specimens were killed half an hour after feeding and at hourly intervals terminating 6 hours after the meal. The last specimens examined were those with constant access to food without a previous fast. Generally, at least two different cytological methods were used simultaneously both for Golgi and mitochondrial preparations. For mitochondria the best results were obtained with Regaud's technique, the tissues being fixed in a refrigerator. Meves's mixture, according to the following formula, gave the next best results: 0·5 per cent. chromic acid in 1 per cent. sodium chloride solution, 15 c.c.; 2 per cent. osmium tetroxide, 3 c.c. The fixing fluid was changed after 4-5 hours, and further fixation continued for 48 hours. Some other methods were also tried. In order to demonstrate the Golgi material, Mann-Kopsch technique was used according to the original formula or with modifications. The methods of Kolatchev and

Sjöval and the silver methods of Aoyama and Da Fano (in spite of less constant results with the latter) proved useful controls. The material was embedded in paraffin. Sections were cut at 5μ and 7μ in thickness. For general histological purposes Bouin and Zenker-formalin proved the most useful fixatives. Golgi preparations as a rule were mounted unstained or in a few cases counter-stained with Altmann's acid fuchsin and differentiated in 90 per cent. alcohol instead of in picric acid. Staining methods for mitochondria consisted of Heidenhain iron haematoxylin, Regaud's haematoxylin, always optimal for the Regaud's preparations, Altmann's acid fuchsin, and Bensley's acid fuchsin light green. Southgate's mucicarmine was used for the demonstration of mucus.

The various fixatives used did not give equally good results with different parts of the alimentary tract. In the case of the stomach, fixatives containing osmium tetroxide revealed many more secretory granules than the other techniques employed; consequently these fixatives are of little use for the study of the mitochondria of zymogenic cells. Observations on the Golgi material of the zymogenic cells were based exclusively on silver impregnated material (Aoyama and Da Fano), as all attempts to obtain good osmic preparations of these cells were unsuccessful. Aoyama's method gave the best results when the tissue was fixed for 4–5 hours. As regards the intestine, the best results were obtained by taking samples from the part situated a short distance above and below the line marked by the descending bowel contents. This routine was carried out in all fed specimens.

<div align="center">OBSERVATIONS</div>

<div align="center">*Proventriculus*</div>

Highly differentiated groups of cells compose the lining of the simple tubular glands and the surface of the gastric lumen. It is therefore necessary to give a separate description of the different types of gastric cells. The zymogenic cells in the tubular glands (Text-fig. 1, figs. 1–4; Pl. I, fig. 16) differ markedly from the superficial columnar epithelium (Text-fig. 1, figs. 5–7); the latter are very similar in morphological outline to those of the intestinal epithelium. Over the short distance which separates the mucous neck cells from the zymogenic cells which line the glandular crypts are scattered cells which cytologically resemble the outer epithelium and, in gross morphological outline, the zymogenic cells. These cells more closely resemble the chief neck cells described by Okanishi (1933) than the parietal cells of mammals. These transitional neck cells have not been identified during the present investigations in the deeper parts of the glandular crypts, and no reference to them was found in the accessible literature on the avian stomach.

Zymogenic Cells

Various names are used to describe the zymogenic cells, e.g. the main cells of the gastric glands (Eklöf, 1914), oxyntic cells (Chinese workers), and zymogenic cells (British workers).
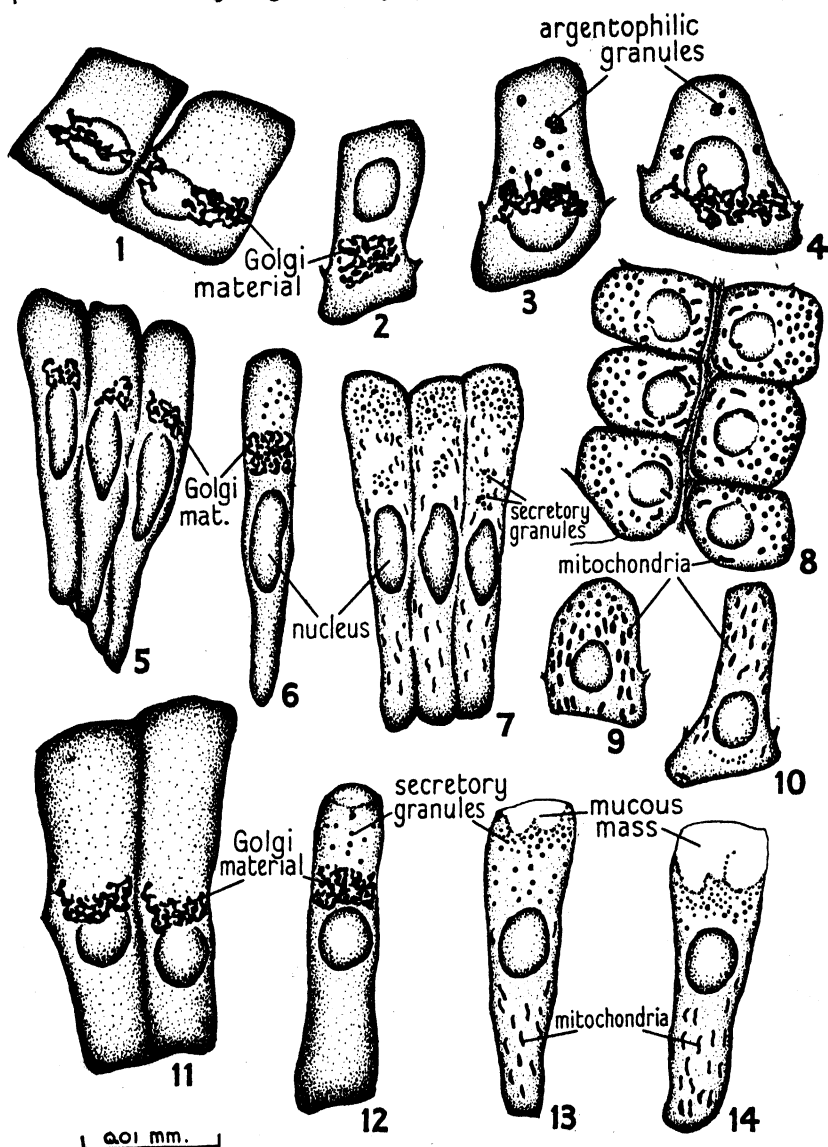
TEXT-FIG. 1.
All figures from proventriculus.

Figs. 1–4 from Aoyama preparations; 5–6 and 11–12 from Kolatchev preparations; Figs. 7–10 and 13–14 from Regaud preparations.

Fig. 1. Zymogenic cell showing Golgi material; after 24 hours' fast.

Fig. 2. Zymogenic cell 1 hour after feeding, cell elongated, apparent reversed polarity of Golgi material.

The zymogenic cells are arranged in a single layer which lines the simple tubular glands along their whole length. Their shape varies, depending on the degree of functional activity, from low cuboidal to elongated columnar cells. In longitudinal sections of the gland these cells are arranged obliquely with their long axis directed slightly towards the opening of the gland. They are arranged in rows in such a way that their distal half, or more than two-thirds of the cell, is not in contact with the corresponding part of the neighbouring cells; thus straight canaliculi are formed between the lateral sides of neighbouring cells (Text-fig. 1, fig. 1, Pl. I, fig. 16–y). This gives longitudinal sections a serrated edge formed by the distal parts of the cells bulging into the lumen of the glandular crypt.

The nucleus is spherical to ovoid in shape and its position depends on the functional stage of the cell; it may lie close to the basal membrane or towards the central region. In all phases of fasting and digestion, most of these cells are densely packed with large, spherical, secretory granules. The number of granules increases considerably during fasting, and in the later stages they extend from the end next to the lumen to the basal part of the cell where they form a mass of closely packed granules which greatly hinders cytological observations. After 24 hours' fast, when there is a maximum accumulation of secretory granules, the cells assume a more cuboidal form with smooth, rounded outlines. The nucleus is spherical and is situated close to the basal membrane. The intercellular canaliculi are difficult to follow, as the lateral cell membranes, under the pressure of accumulated secretion, are brought into proximity with their neighbours (Text-fig. 1, fig. 8). Commencing half an hour after the ingestion of food the number of secretory granules decreases considerably and the outline and shape of the cell become altered. The cell becomes thinner, elongates, and assumes an angular outline. The nucleus moves closer to the central region of the cell and assumes an ovoid shape (Text-fig. 1, fig. 2). The intercellular canaliculi now appear as incisions or long narrow clefts wedged between the cells; this is due to the contraction of the lateral cell borders. The evacuation of the cells, which starts with the intake of food, increases markedly until 3 hours after a meal. In sections prepared for the study of the mitochondria, unstained vacuoles appear in

Figs. 3 and 4. Zymogenic cells 2 hours after feeding; argentophilic granules present in cytoplasm.

Fig. 5. Cell of surface epithelium showing Golgi material; after 24 hours' fast.

Fig. 6. Cell of surface epithelium, 1 hour after feeding, Golgi material enlarged; secretory granules seen above it.

Fig. 7. Cell of surface epithelium showing mitochondria and secretory granules.

Fig. 8. Zymogenic cells showing mitochondria and secretory granules; after 24 hours' fast.

Figs. 9 and 10. Zymogenic cells 1 hour after feeding; showing mitochondria and secretory granules below the nucleus in fig. 10.

Fig. 11. Mucous neck cells showing Golgi material; after 24 hours' fast.

Fig. 12. Mucous neck cell 1 hour after feeding; Golgi material enlarged and secretory granules seen above it.

Figs. 13 and 14. Mucous neck cells 1 hour after feeding; showing mitochondria, secretory granules, and formed mucous mass.

the place of secretory granules. Three hours after feeding the cells begin to refill with secretory granules, and return to their former shape. The number of granules gradually increases, and 6 hours after the intake of food reaches the level usually observed in birds with constant access to food, which is never as great as in fasting specimens.

It must be noted that only a certain number of cells is involved in the secretory process at the same time, and that each cell appears to act as an independent unit. During the first hours of digestion, cells situated close together often show marked differences in shape and functional stages. Cells at the bottom of the glandular crypts appear to be the least involved in secretion and contain fewer secretory granules than any of the other cells.

Golgi material. The Golgi material in zymogenic cells was studied exclusively in material impregnated with silver (Aoyama and Da Fano). Certainly these methods are much less delicate than osmic methods, but when carefully handled they provide much valuable information. No great difficulties were encountered with silver methods and the Golgi material was shown in cells along the whole length of the glandular canal. Cells in the middle of the glandular tubule are more difficult to impregnate than those nearer the bottom and neck of the crypt; this is due to the large number of secretory granules present in the cells of the middle region.

The Golgi material in zymogenic cells consists of thick filaments, twisted and joined in a more or less compact manner. It always lies in the basal part of the cell exactly at the level of the bottom of the intercellular canaliculi (Text-fig. 1, fig. 1; Pl. I, fig. 16). After 24 hours' fast, when the cells are packed with secretory granules and the nucleus is close to the basal membrane, the Golgi material surrounds the nucleus to form an equatorial belt (Text-fig. 1, fig. 1). The elements of the Golgi material seem to be closer together than in actively secreting cells. With the decrease in the number of secretory granules, subsequent to feeding, the cell elongates and the nucleus moves towards the central region. The Golgi material does not move with the nucleus and thus gives what students of the theory of polarity would describe as 'reversed polarity'. At this phase there is a considerable loosening of the Golgi elements towards both poles of the cell. The Golgi material, however, never moves from its original level (Text-fig. 1, fig. 2; Pl. I, fig. 16–x.)

In the next phase, elongate cells, with Golgi material situated below the nucleus, show changes which strongly suggest that secretory activity has begun. Small clusters of argentophilic granules are present above the Golgi material in the supranuclear zone (Text-fig. 1, figs. 3–4). In deeply impregnated material the granules are blackened and appear as a uniform mass, but in lightly impregnated material the clusters of granules are clearly shown, and the faintly brown outline of each granule is visible. This argentophilic material was observed in many cells 1–2 hours subsequent to feeding; after that time they were less frequently seen and were seldom observed 3 hours after feeding. It is probable that these granules migrate from the Golgi zone during increased cellular activity, and represent a stage in the formation of
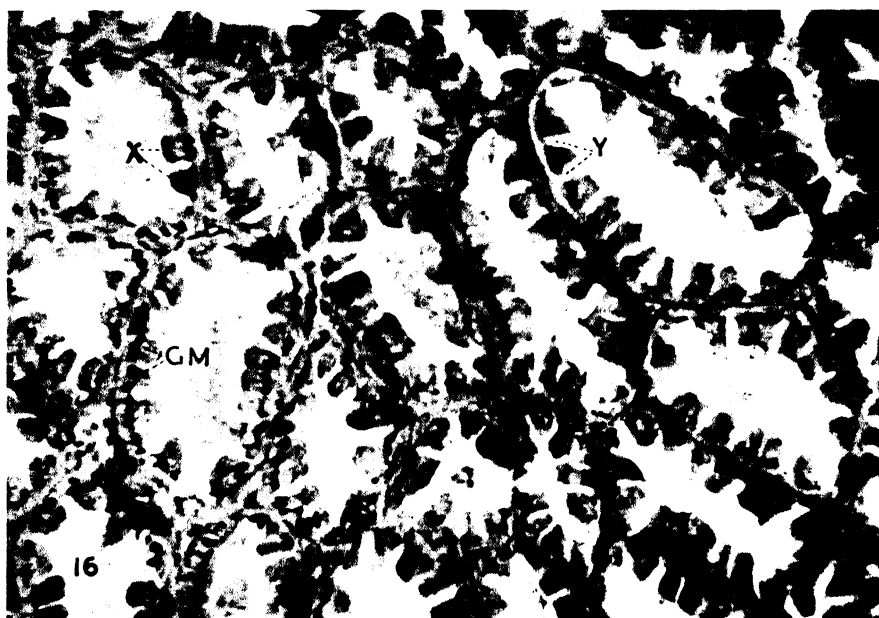
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14

15

the secretion granules. As they are visible for a short time after feeding (1–2 hours) and are not present at any other phase, it is probable that a rapid increase in secretory activity leads to the freeing of products which are not yet fully formed and possess the argentophilic properties of the Golgi material itself, and that their transformation into granules of secretion takes place in the cytoplasm. Owing to the coarse outlines of the Golgi material (silver methods), it is impossible to identify secretory granules amongst the Golgi material. The earliest secretory granules are visible in mitochondrial preparations and are restricted to the Golgi field (Text-fig. 1, fig. 10).

Present observations on zymogenic cells support the view that the situation of the Golgi material, at least in these cells, is not influenced by the relative position of the nucleus. The apparent reversal of polarity, which has aroused much discussion, is of short duration and is produced by a free movement of the nucleus and not of the Golgi material.

The location of the earliest secretory granules in the Golgi field, the presence of clusters of argentophilic material which migrates from the Golgi zone during the later phase of secretion, and the changes in the form of the Golgi material indicate that the Golgi material participates in secretory activity.

Mitochondria. As Regaud's haematoxylin tends to stain mitochondria only, clear pictures of the mitochondria were obtained in zymogenic cells from fasting and fed specimens prepared by this method.

Short rod-shaped mitochondria are numerous; in addition a few long thick filaments, of equal diameter, are present. The small number of granules observed would suggest that they are cross-sections of rods and filaments. A few swellings situated at intervals along the mitochondria are sometimes visible. In cells densely filled with granules the majority of the mitochondria are situated in the basal half of the cell, but a few are also dispersed in the supranuclear zone and are visible between the secretory granules (Text-fig. 1, fig. 8). At this phase the mitochondria are arranged more or less at random without a special polar orientation.

At the time when the elongate cells enter upon secretory activity, the mitochondria become distributed throughout the cytoplasm, but the majority are in the supranuclear zone. Polar orientation is strongly marked by the arrangement of the rods parallel to the long axis of the cell (Text-fig. 1, figs. 9–10). In the early phases, very small and deeply stained granules (Regaud's haematoxylin) are observed below the nucleus in the region occupied by the Golgi material (Text-fig. 1, fig. 10). These granules are much smaller than cross-sections through rod-shaped and filamentous mitochondria, or sections through mature secretory granules. Their size, form, and location strongly suggest that they are the early stages of secretory granules. Unfortunately, no stages intermediate between these granules and the mature granules of secretion were found. During the stage when the detached argentophilic material is present in the supranuclear region (1–2 hours after feeding), it would appear that the total number of mitochondria is considerably greater than in the cells of fasting animals. It is unfortunately difficult to determine

whether there is a real increase in number or whether many of the mito-
chondria are hidden by secretory granules, and, therefore, invisible until
there is a considerable decrease in the number of granules. No evidence
suggesting the division of the mitochondria or their direct participation in
secretion was observed.

Mucous Neck Cells

These are large columnar cells, cylindrical in shape, resembling to some
extent goblet intestinal cells. The supranuclear pole is filled with mucus
formed into a goblet, but without distending the cell as in the case of the
intestinal goblet cells. The Golgi material, easily demonstrated by both
silver and osmic methods, forms a cup-like structure adjacent to the lower
border of the accumulated mucus (Text-fig. 1, fig. 11; Pl. I, fig. 15). Mito-
chondria are present as extremely fine filaments which are shown most
clearly in material fixed according to the method of Meves and stained with
acid fuchsin. Their number is always very limited and they are present
almost exclusively in the subnuclear cytoplasm (Text-fig. 1, figs. 13–14). The
secretory cycle and accompanying changes are analogous to those described
by other workers for goblet intestinal cells, with the exception that the secre-
tory granules are extremely small and fill all the supranuclear zone. The
granules show a marked affinity for acid fuchsin, but not for haematoxylin.
These numerous granules gradually accumulate at the glandular pole of the
cell where a uniform mass of mucus secretion is formed and extends towards
the Golgi zone (Text-fig. 1, fig. 14). The characteristic reaction for mucus
is given by the uniform mass, but not by the small granules. During a fast,
practically all these cells are filled with secretion. The elimination of mucus
starts with the intake of food, and follows a rather peculiar course. The
outer masses of secretion are passed into the lumen where they may be
easily demonstrated. Elimination is never complete, and the lower part of
the mucous mass is immediately replaced with newly produced secretion. The
Golgi material, during increased secretory activity, expands and increases in
volume (Text-fig. 1, fig. 12). With the accumulation of secretion it moves
closer to the nucleus and becomes smaller. No noticeable changes in shape,
location, or in the number of mitochondria were observed in any of the
preparations.

Surface Epithelium

The surface cells, bordering the gastric lumen, are of the regular, simple
columnar type. The nucleus is oval and lies in the middle of the cell. Numerous
granules, spherical in shape, fill the supranuclear region. They vary in num-
ber, depending on the functional stage, but are always present during fasting
and after feeding (Text-fig. 1, fig. 7). As in the mucous cells, these granules
stain more readily with acid fuchsin than with haematoxylin.

The Golgi material is a complicated structure which resembles a network,
and lies between the nucleus and the accumulated secretory granules at the

glandular pole (Text-fig. 1, fig. 5). The mitochondria, very minute in diameter, and almost exclusively filamentous, are scattered more or less evenly in the supra- and subnuclear cytoplasm. They are few in number, especially in comparison with those of the intestinal epithelium (Text-fig. 1, fig. 7). Immediately after feeding the number of secretory granules decreases markedly, while simultaneously new granules originate in the Golgi zone and are scattered through the supranuclear region (Text-fig. 1, figs. 6–7). Within 2 hours after feeding the accumulation of small granules, close to the distal cellular margin, is as dense as in the fasting condition. Owing to their minute diameter, the mitochondria are very difficult to observe, and no marked changes in their form or disposition were noted.

The neck cells which lie between the mucous neck cells and the zymogenic cells are transitional forms, and in outline resemble the zymogenic cells. They show exactly the same internal cytological structure as the surface epithelium, and the description of the latter applies equally to these transitional cells.

Gizzard

The gizzard is a peculiar structure and in many respects differs markedly from the proventriculus. Its mucous membrane has a thick, horn-like inner lining, which is generally believed to be a secretory product of the epithelium lining the tubular crypts.

The epithelial lining consists of a single layer of cuboidal cells arranged in protruding lamellae which form simple elongated crypts. These are filled with keratinoid material which is connected with the outer horn-like cover. The cuboidal cells near the apices of the lamellae are more elongated, and often club-shaped, with a wider part directed towards the outer keratinoid mass. A very large spherical nucleus generally fills more than half the cell, so that the latter often bulges into the lumen of the crypt. The epithelial cells which line the crypt are strikingly different from other gland cells. Their peculiar behaviour suggests that they belong to a separate group of cells and resemble more closely epithelia undergoing keratinization, first described by Deineka (1912).

Examination showed that only the cells at the bottom of the crypts and the lower parts of the lamellae are normal cells in which all the components are visible. Proceeding from the bottom of the crypt to the apices of the lamellae, the cells show progressive degenerative changes which finally lead to their death. In material fixed by Meves's method and stained with acid fuchsin-picric acid, whole cells embedded in the lower parts of the keratinoid mass are clearly seen. Forty-eight hours' fixation in Meves's fluid is sufficient for the osmium tetroxide slightly to darken the numerous keratinoid granules which fill the cells as well as the intercellular spaces between the dead cells. The cells embedded in the keratinoid mass seem to be preserved by the latter. The keratinoid cover is not a structureless mass, but consists of numerous granules, dead cells, and a more or less uniform cementing mass

which stains a deep yellow with picric acid. The more superficial layers, however, are stained a uniform yellow and do not show any structural details.

In the cells of the lower parts of the crypts the Golgi material is a reticulate structure similar to that of the other gland cells. It lies at one pole of the nucleus, or, collar-like, surrounds the middle region of the nucleus (Text-fig. 2, figs. A, B). A few small granules are seen scattered throughout the twisted filaments of the Golgi material. In the cells situated towards the apex
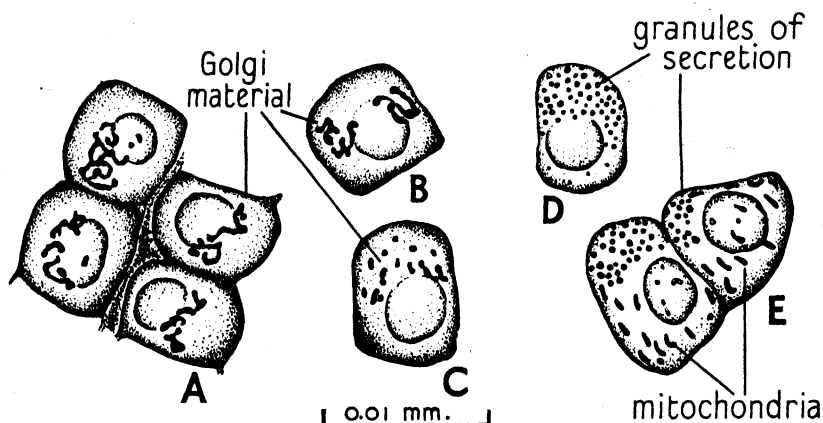
TEXT-FIG. 2

Figs. A and B. Cells from the bottom of the crypt, with Golgi material.
Fig. C. Cell near the top of lamellae, with Golgi material breaking up.
Fig. D. Cell near the top of lamellae, with granules of secretion.
Fig. E. Cells with mitochondria and granules of secretion.

of the lamellae, the Golgi material is less voluminous and is progressively broken up into short rods and granules (Text-fig. 2, fig. C). In the club-shaped cells, on the apices of the lamellae, Golgi material is not revealed by any of the methods used. In the nuclei of cells situated towards the apex of the lamellae there is a marked increase in the number of darkly stained granules. In the vicinity of the top of the lamellae the nuclei appear to shrink, and the nucleoplasm is uniformly darkly stained.

Rod-like mitochondria, oriented parallel to the long axis of the cell, are present in cells in which the Golgi material is well developed; as a rule they are less numerous than in the other gland cells (Text-fig. 2, fig. E). In cells towards the apex, where the Golgi material is breaking up, the mitochondria are less numerous and finally disappear.

Secretory granules are few in number in the basal cells and are present in the supranuclear zone. They gradually increase in number in the cells situated nearer the top of the lamellae (Text-fig. 2, fig. D). In the cells in the vicinity of the apex of the lamellae, in which the Golgi material is breaking

up, the granules reduce osmium tetroxide; consequently they assume a yellow-brown colour like the surrounding keratinoid mass.

Fasting and feeding do not induce cytological changes in any of the cells of the gizzard. The formation of the granules seems to be progressive and continuous. The few granules present in the cells at the bottom of the crypt, and the relatively small amount of Golgi material which does not show any changes during the phases investigated, indicate that the formation of the secretion is very slow and is not influenced by the process of digestion. Expulsion of the secretory granules must take place more slowly than production, so that the number of granules gradually increases within the cell. The process of secretion appears to slow down in the older cells at the top of the lamellae and finally leads to their exhaustion and death. These cells do not disintegrate, but become embedded in the secretory product.

Fragmentation and terminal disappearance of the Golgi material and of the mitochondria are no doubt signs of the degenerative processes taking place within the cell. The few mitotic figures seen in some of the material at the bottom of the crypts may indicate the method of replacement of old cells. As elimination of the old cells is slow, cell division resulting in new cells is of infrequent occurrence.

The slow secretion of the epithelial cells, independent of digestion, indicates that their function is to renew the keratinoid cover worn away by the mechanical function of the gizzard.

Intestinal Epithelium

As regards histological classification and description of the cells of the intestinal epithelium, there is practically nothing to add to the detailed works of Clara (1926–7). The cells which compose the intestinal lining are arranged in a single columnar layer. In addition to the main epithelial cells (with epithelial cells of the Lieberkühn crypts as their variant) and goblet mucous cells, two kinds of cells enter into the composition of the intestinal lining; these are the chromaffine and Paneth cells.

The main epithelial cells are referred to in this work as epithelial cells, and are the most numerous type. The goblet cells, scattered between the epithelial cells, gradually increase in number from the duodenum to the rectum, where they are nearly always predominant and in some cases may form the entire intestinal lining. The number of goblet cells present in a particular region depends on the physiological phase; they increase during a fast and during the later stages of digestion, but diminish considerably in the first hours after the intake of food. The goblet cells are numerous in the crypts and diminish in number towards the top of the intestinal villi.

Epithelial Cells

These cells vary in shape and size, depending upon their situation and the degree of contraction of the intestinal villi. The oval nucleus is situated in the basal half of the cell, but is usually closer to the middle of the cell

than to the basal pole. The outer pole of each cell has a characteristic structure known as the striated border. It is a well-marked, girdle-like extension of uniform texture with delicate longitudinal striations; this region is free of cytoplasmic components. The cell membrane is very faintly marked.

The epithelial cells of the Lieberkühn crypts differ from those of the villi. The nucleus is large and is situated close to the basal membrane. A striated border is absent, and the cytoplasm has a much stronger affinity for dyes, such as haematoxylin and acid fuchsin, than that of the cells in the villi. This property creates some difficulties in cytological work, and demands careful handling during staining and differentiation.

In addition to the typical epithelial forms, a number of narrow, darkly stained cells are visible. Clara (1926) suggested that these are normal epithelial cells compressed by neighbouring goblet cells, or that they are goblet cells subsequent to the discharge of their mucous content. During the present investigation, it was noted that these cells become more numerous soon after feeding, while the goblet cells diminish considerably in number, and that they are very seldom present between the goblet cells. This strongly supports Clara's second suggestion that the dark cells are regenerating goblet cells. Mitotic divisions are always present, but are found almost exclusively in the crypts.

Little need be said regarding the morphology of the resting cells of specimens killed after 24 hours' fast, except that the basal half of the cell appears to be more conically compressed than during digestion. In sections stained for mitochondria, no granules other than granular mitochondria, or cross-sections of rods and filaments, are present. After feeding the cell appears to be more uniform in width and the nucleus seems to move slightly from its central position towards the basal third of the cell. A light area without any distinct border becomes visible in the supranuclear region, and increases in sharpness and size as digestion proceeds. It corresponds closely with the area occupied by the Golgi material and is the negative image of the latter. In the rows of cells on a villus the areas form a lighter zone above, and parallel to, the anterior limit of the nuclei. Secretory granules, scattered in and above the Golgi field, are visible and increase in number during the phases of secretory activity (Text-fig. 3, figs. 9–10). There appears to be no fundamental difference in the behaviour of the cells of the Lieberkühn crypts. Here also secretory granules appear in the Golgi field.

Golgi material. After feeding, the response of the Golgi material was found to vary greatly at different levels of the intestinal canal. The best results were obtained by the examination of sections of the canal immediately above and below the line marked by the descending bowel content. The duodenum and the upper part of the ileum gave the quickest and most marked response, and are, therefore, the most favourable regions for observations.

After a 24 hours' fast the Golgi material in the epithelial cells throughout the entire length of the intestine shows approximately the same morphological

pattern. The Golgi material occupies a median position between the nucleus and the lumen; in this phase it is very small and consists of a few thin rods and threads with granular swellings of variable size, usually not more than twice the thickness of the threads themselves. The rods and threads are arranged more or less parallel to the longitudinal axis of the cell. A few cross-links, usually of very small diameter, connect the longitudinal threads so that the Golgi material appears as a simple elongated reticular structure (Text-fig. 3, figs. 1–2; Pl. II, fig. 13). In this phase, the Golgi material is most difficult to impregnate successfully. The Mann-Kopsch method gave the most satisfactory results, but the tissue must be kept for over 3 weeks in 2 per cent. osmium tetroxide at room temperature. Prolonged osmication is needed for the other osmic methods. Silver impregnation may be carried out for the usual length of time, but unfortunately, owing to the many silver deposits, may be used only as a control.

After feeding, parallel with the movement of the food contents inside the bowel wall, marked changes in the Golgi material quickly take place. At the time when food material reaches the duodenum numerous secretory granules, arranged in compact clusters and longitudinal rows, appear in the Golgi region. The clusters create the illusion that fragmentation of the Golgi material takes place (Text-fig. 3, figs. 3–4; Pl. II, fig. 14). This phase is of short duration and mature granules soon begin to move away from the Golgi field. At the same time the Golgi material begins to increase markedly in size and its reticulate structure becomes more evident. It is now a complicated structure with numerous granules situated along the connecting links and between the meshes of the network (Text-fig. 3, fig. 5; Pl. II, fig. 15). Many granules which have moved away from the Golgi zone are present above it, and the stages of their development and terminal accumulation below the striated border may be followed with ease. The first cells to respond to stimulation are situated on the top of the villi, and the onset of secretion in the cells near the crypts begins later. This facilitates observation of the consecutive phases of secretion, which may be followed in a single villus. Approximately 1 hour after feeding, the Golgi material of the cells of the duodenum is a strong reticular structure stretching between the lateral cell borders. In neighbouring cells it is always at the same level and its position is not influenced by the distance of the nucleus from the basal pole of the cell; consequently, longitudinal sections across the villi show a wide, deeply impregnated belt situated parallel to and above the nuclei. Numerous granules in the supranuclear region give this part of the cell a much darker appearance than the remainder of the cytoplasm (Pl. II, fig. 15).

Analogous changes were easily followed in the proximal parts of the ileum, parallel with the progress of the bowel contents. These changes are less striking towards the terminal part of the small intestine, and in the lower part of the ileum they are scarcely, if at all, noticeable. In the caeca and rectum no changes were observed. The maximum manifestation of the stimulating action of food seems to be about 1 hour after direct contact with the contents
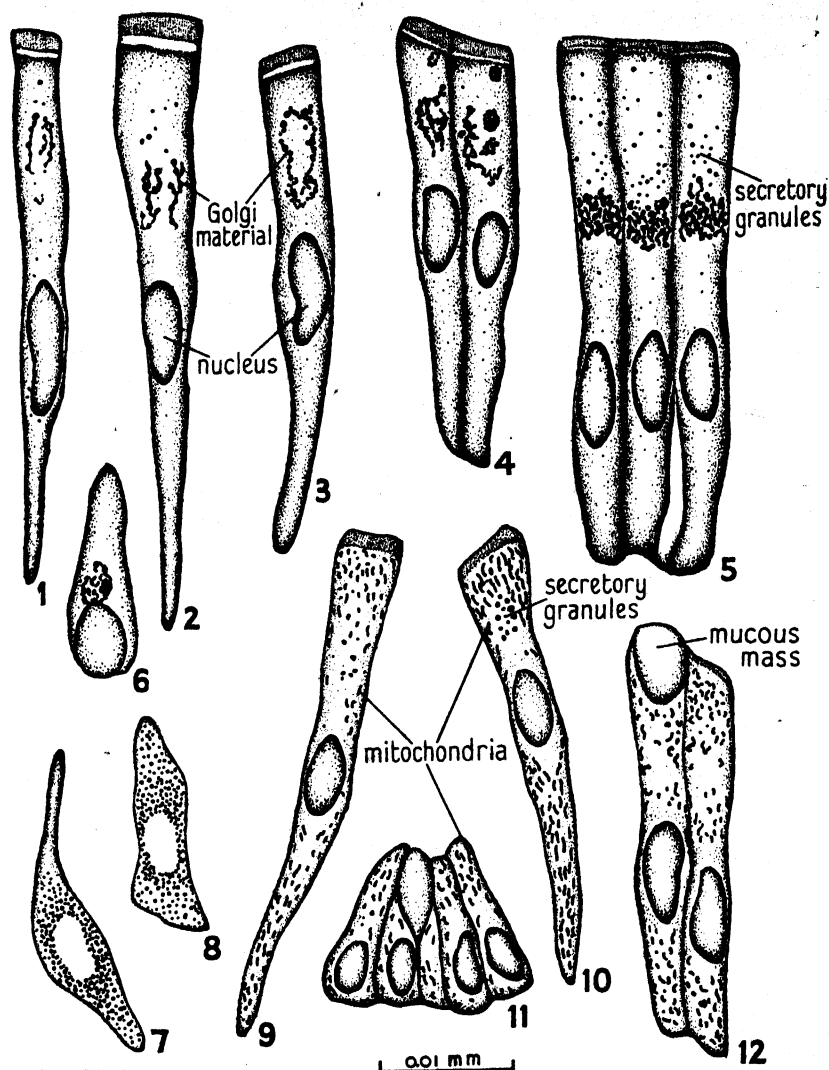
TEXT-FIG. 3.

All figures of intestinal epithelium.

Figs. 1–6 from Mann-Kopsch preparations; 7–12 from Regaud preparations.

Figs. 1 and 2. Cells from villi, showing Golgi material; after 24 hours' fast.

Figs. 3 and 4. Cells of the upper part of intestine upon direct contact with food; fragmentation of Golgi material and new secretory granules.

Fig. 5. Cells of duodenum 1 hour after feeding; Golgi material hypertrophied and secretory granules shown above it.

Fig. 6. Cell of glandular crypt showing Golgi material; after 24 hours' fast.

Figs. 7 and 8. Chromaffin cells.

of the intestine. From that time slow retrogressive changes set in. In speci-
mens which had constant access to food the shape of the Golgi material varies
little, and is intermediate between that of resting and active cells of specimens
previously starved.

Besides the morphological changes, physico-chemical changes appear to
take place in the Golgi material. With the onset of secretory activity there
is a marked increase in the power of the Golgi material to reduce osmium
tetroxide; therefore a quicker and deeper blackening of the Golgi material is
a visible manifestation of these changes. As already pointed out, resting cells,
fixed in Mann-Kopsch, must be kept in osmium tetroxide for 3 weeks in
order to give satisfactory results. In cells previously brought to the resting
phase by fasting and subsequently stimulated by direct contact with food,
the Golgi material is blackened after 2 weeks in osmium tetroxide.

In cells prepared by osmic methods very small impregnated granules
(mostly on the border of microscopic visibility) are present in various parts
of the cytoplasm. In some cells a small agglomeration of granules is visible
just below the nucleus. In the nuclear region, where only a narrow strand
of cytoplasm lies between the cell membrane and nuclear membrane, the
granules seem to lie on the surface of the nucleus and are deeply impregnated.
The presence of granules below the nucleus in some cells suggests that their
passage from the basal part of the cell towards the outer pole is obstructed
by the nucleus. In the Golgi zone they are intermingled with the larger
secretory granules. The granules increase in number during the secretory
phase; this suggests that they may be the prototypes of secretory granules
which at a later stage are intimately connected with the Golgi material.
Further investigation on the nature of the granules is desirable.

Mitochondria. In their distribution and arrangement the mitochondria of
the intestinal epithelium of the domestic fowl follow the general pattern
described by many investigators of vertebrate material. The anterior intesti-
nal cells, where secretory response is most pronounced, were chiefly used
for examination. In the resting phase two areas of mitochondrial aggregation
were observed—one immediately below the striated border and the second
in the basal subnuclear region of the cell. The aggregation at the outer pole
of the cell is denser than that in the subnuclear region. In the rest of the cyto-
plasm the mitochondria are less numerous and are fairly equally distributed;
they are practically absent from a small area directly above and below the
nucleus (Text-fig. 3, figs. 9–10). All forms of mitochondria are present, but
wavy and slightly curved filaments of various lengths predominate. Short
rods and granules, though few in number, are also encountered. Short

Fig. 9. Cell from villus of upper part of intestine after 24 hours' fast; to show mito-
chondria.
Fig. 10. Cell from villus of the upper part of intestine half an hour after feeding; to
show mitochondria and secretory granules in Golgi field.
Fig. 11. Cells of glandular crypt after 24 hours' fast; to show mitochondria.
Fig. 12. Cells of caeca; mitochondria irregular in shape and arrangement.

filaments and rods appear to be more characteristic of the subnuclear zone, where only a few granules are seen. Polar orientation of the mitochondria parallel to the long axis of the cell is already well known and has been described by most workers on the intestinal epithelium.

Soon after the intake of food, at a time which closely corresponds to the first visible secretory response of the Golgi material, the mitochondria undergo certain changes. The more deeply stained segments, and bleb-like swelling which are present during the resting phase, disappear and evenly stained forms predominate. The agglomeration of the mitochondria close to the striated border becomes less marked and a more even distribution is observed in the supranuclear zone. Short and granular forms appear to diminish in number but do not entirely disappear. A characteristic feature of the mitochondria in this transitory phase, which is of shorter duration than the analogous period in the history of the Golgi material, is a decreased affinity for ordinary dyes such as acid fuchsin and haematoxylin. Consequently, more skill and care are needed in differentiation than during the other stages. It seems that Saito, describing his 'primary mitochondria', unwittingly referred to this phase in which the mitochondria are most difficult to stain. Secretory granules appear in the Golgi area which now contains few mitochondria and is visible as a light zone. While the small early granules stain deeply, the older ones, which become rather vesicular, stain very faintly, but are shown most successfully in sections stained with acid fuchsin. Subsequently to this phase, the mitochondria exhibit a greater variety of form; their tortuous lines make them similar to those described for the resting phase.

Towards the posterior part of the alimentary tract, the mitochondria of the epithelial cells show greater variability; the threads are less regular and do not show such a marked polar orientation as in the duodenum. Bleb-like swellings, granular and ring-like forms are more numerous (Text-fig. 3, fig. 12). The response of the mitochondria in the lower parts of the intestine is slower and decreases progressively towards the end of the small intestine. In the cells of the crypts of Lieberkühn, shorter filaments predominate over the other forms, and the mitochondria appear to be less numerous than in the cells of the villi (Text-fig. 3, fig. 11). No change of form or disposition was noted in the mitochondria of these cells.

Goblet Cells

The present account does not provide much new information on the goblet cells, and most of the observations made confirm the descriptions of previous workers on other animals.

The goblet cells of the domestic fowl differ markedly from those of mammals. They are always in the form of a neatly shaped goblet. The lower part, which contains the nucleus and most of the cytoplasm with its components, is narrow. The nuclei of the goblet cells usually stain more deeply than those of epithelial cells. The Golgi material is situated between the

nucleus and the lumen and forms a network composed of rods and filaments of variable thickness (Text-fig. 4, figs. 1–6).

In the early phases of secretion the Golgi material is present, for the most part, in the form of short segments and threads (Text-fig. 4, figs. 1–2). With the onset of secretory activity the reticulum becomes much more complicated, forming a cylindrical basket which stretches throughout the whole length of the narrow part of the cell. Secretory granules are visible in intimate connexion with the rods and threads (Text-fig. 4, figs. 3–4). Free osmiophilic granules are seen inside the basket intermingled with non-osmiophilic vesicles. The non-osmiophilic vesicles give a positive reaction with mucus-staining dyes, such as mucicarmine, toluidine blue, and thionine; they migrate from inside the Golgi basket to the outer pole of the cell where they gradually fuse together to form a uniform mass of mucus which replaces the cytoplasm and pushes the cytoplasmic components towards the narrow basal region (Text-fig. 4, figs. 5–6).

Consecutive phases in the production of mucus may be traced by comparing Golgi preparations with material prepared for the demonstration of mitochondria and counter-stained with mucicarmine. As the formation of the goblet nears completion, the secretory process seems to diminish, and the Golgi material begins to return to its original form. Free secretory granules appear to be short-lived and their transformation into non-osmiophilic mucous vesicles must be rapid. The mitochondria are similar in form to those of the intestinal epithelial cells (Text-fig. 4, figs. 7–12). They are pushed towards the narrow basal part of the cell by the accumulation of the secretory mass in the goblet, and are most numerous close to the membrane separating the cytoplasm from the basal border of the mucous goblet (Text-fig. 4, figs. 11–12). No conclusive evidence was obtained suggesting their direct transformation, or even participation, in the secretory processes.

Chromaffin and Paneth Cells

Only one type of these cells was observed in the material investigated. This type is present throughout the intestine and is most numerous in the crypts. In form, these cells vary from bottle-shaped, with the narrow neck directed towards the intestinal lumen, to spindle-shaped cells lying between the epithelial cells. The large lightly stained vesicular nucleus lies in the central part of the cell (Text-fig. 3, figs. 7–8).

The cytoplasm is filled with a considerable number of granules possessing peculiar properties. In mitochondrial preparations they stain deeply and uniformly with haematoxylin. In impregnated material they blacken uniformly with osmium tetroxide, and as easily as the Golgi material during secretion. During fasting and at all times after feeding the cells behave in the same way and no differences in the number of granules, or in their staining properties, were noticed.

The function of these cells remains obscure, and the present observations do not provide any new information.
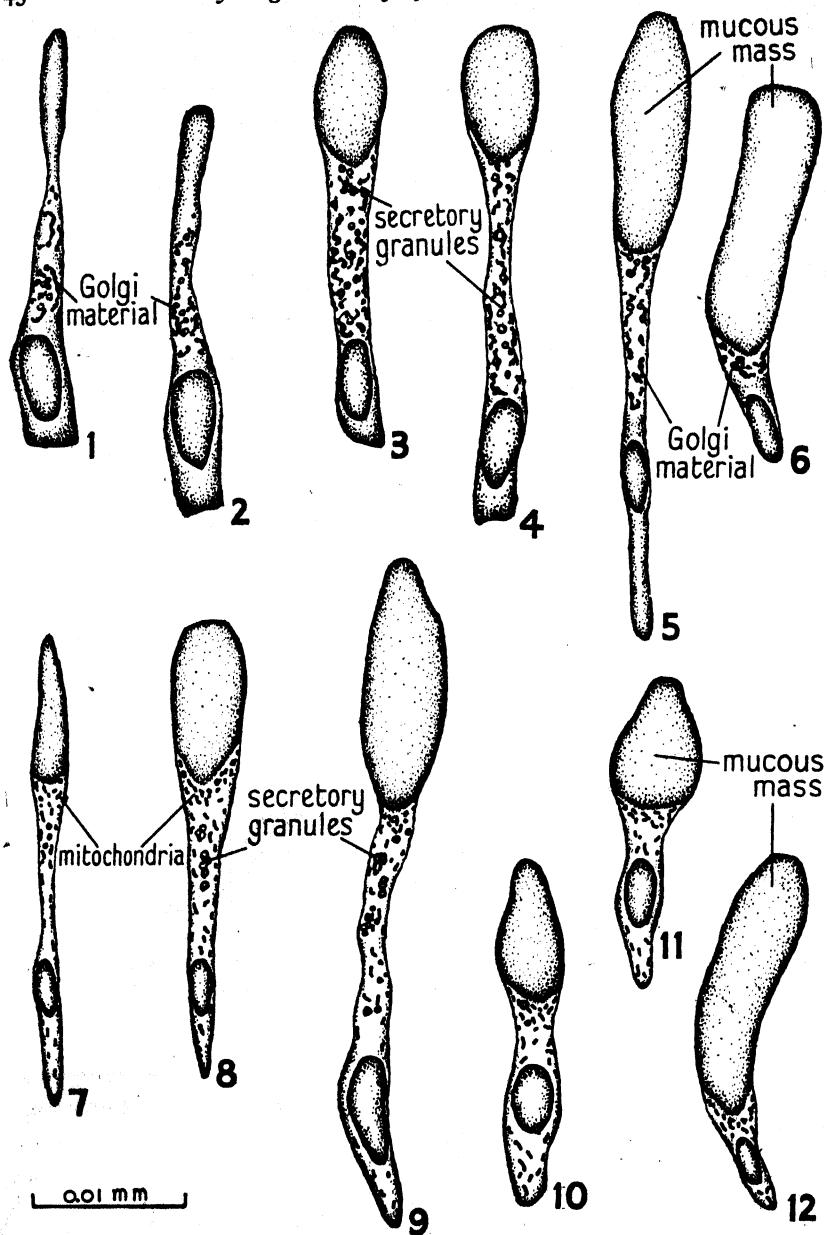
TEXT-FIG. 4.
All figures show secretory cycle in intestinal goblet cells.
Figs. 1–6 from Mann-Kopsch preparations; 7–12 from Regaud preparations.

DISCUSSION

Proventriculus

The present account of the gastric epithelia of the fowl supplements previous inadequate cytological studies on the avian stomach, and provides certain information not hitherto recorded.

The present investigation demonstrates that the cytoplasmic components of the different types of gastric cells undergo marked changes during periods of fasting and after feeding. The changes observed vary considerably in the different types of cells; they are most prominent in the zymogenic cells and least striking in the cells of the surface epithelium. In all types of gastric cells the maximum accumulation of secretory material occurs after a 24 hours' fast. Subsequently to feeding there is a gradual decrease in the amount of accumulated secretory material, and the progressive evacuation, which begins immediately after food intake, reaches its peak about 2–3 hours after a meal. After that time there is a gradual increase in the amount of secretion present in the cells. A complete evacuation of secretory material does not take place at any time after feeding. All the gastric cells appear to exhibit constant secretory balance with variations depending on the intake of food and periods of fasting.

As regards the cytoplasmic components it is of interest to note that the response in the gastric cells, due to feeding, is noticeably less marked than in those of the intestinal epithelium. This fact, as well as the constant presence of secretory material in all the gastric cells throughout the phases investigated, suggests that during fasting the secretory processes are greatly retarded, but are not completely suppressed and revive after feeding. This suggestion agrees with the observation of Ma, Lim, and Liu (1927), who, using the much more drastic methods of histamine stimulation on dogs with gastric fistula, never succeeded in exhausting the zymogenic cells to such an extent as to free them completely of secretion. Attempts to trace the early stages of secretion in each group of cells indicates that the granules originate in the Golgi field. Parallel with increased production of granules of secretion a moderate hypertrophy of the Golgi material takes place. In the mucous cells and surface epithelium the secretory granules were easily followed from the Golgi material to the outer glandular pole of the cell, but in the zymogenic cells they could only be traced by comparing the impregnated material with sections stained for the demonstration of mitochondria.

Figs. 1 and 2. Cells in the early stages of secretion; Golgi material small, few secretory granules.

Figs. 3 and 4. Cells in advanced stages of secretion; Golgi material hypertrophied, secretory granules more numerous.

Figs. 5 and 6. Cells in terminal stages of secretion.

Fig. 7. Cell in early stages of secretion, showing mitochondria.

Figs. 8 and 9. Cells in advanced stages of secretion; showing secretory granules and vesicles.

Figs. 10–12. Cells in terminal stages of secretion, showing mitochondria accumulated on the border of the mucous goblet.

The Golgi material of gastric cells was investigated by Golgi (1909), who pointed out that it varies greatly in different types of gastric cells. Golgi also directed his attention, and that of many subsequent workers, to the strange location of the Golgi material in the zymogenic cells. D'Agata (1910) attempted to prove that simple traumatic lesions may cause a reversal of polarity of the Golgi material of the superficial gastric epithelium. Giroud (1928), on the other hand, claimed that the equatorial situation of the Golgi material in zymogenic cells is a purely mechanical occurrence due to pressure from the secretory granules. It would seem from the available literature that most of the discussions on the Golgi material in the zymogenic cells and its peculiar topography are based on the original works of Golgi, and on those of Kolster (1913). None of the later workers, except Kopsch (1926) and Hibbard (1942), gave any information on their methods and degree of success in demonstrating the Golgi material of these cells. The present observations agree with the statements of other authors that the behaviour of the Golgi material of zymogenic cells is completely different from that of other glandular cells. The writer, however, believes that previous statements that the Golgi material of the zymogenic cells always lies lateral to the nucleus are inaccurate. A considerable variation in the position of the nucleus in relation to the Golgi material was observed during the present work. At the time when there is a marked accumulation of secretory granules, the nucleus moves towards the basal membrane, and is encircled by the Golgi material in the form of a vertical collar. When the granules decrease in number, the nucleus moves towards the central part of the cell and leaves the Golgi material behind in its former position. The question of the reversed polarity of the Golgi material was a problem which involved much discussion and controversy. The stable position of the Golgi material observed in the present work suggests that in the case of these cells, and perhaps also in other cells, its polarity is purely relative, depending upon the free movement of the nucleus which appears to be independent of the Golgi material. The present observation strongly supports Pollister's conclusion (1938) that the peculiar orientation of the Golgi material in the zymogenic gastric cells is not associated with the nucleus, but is in relation to the zone of discharge of secretion. Pollister stresses further that careful study of the course of the capillaries at the base of these cells makes it clear that the Golgi material is oriented in relation to the course of cytoplasmic flow between the blood capillaries and the secretory surface.

Much work has been carried out on the mitochondria of the zymogenic cells. Perhaps because of difficulties, and in most cases because of failure, to demonstrate the Golgi material the attention of many workers, especially those who looked on the mitochondria as centres of secretion, has been directed to the appearance of these cytoplasmic components in different functional phases. Some of the earlier workers stated that mitochondria are less abundant in loaded zymogenic cells than in those from which the granules of secretion have been discharged (Eklöf, 1914; Tschassownikow, 1927).

The Chinese school of cytologists, following Cowdry's conception, published a series of papers on zymogenic cells in which, with the help of elaborate descriptions, they ascribe to the mitochondria an exclusive, or at least a major, role in secretion (Lim and Ma, 1927; Ma, Lim, and Liu, 1927; Ma, 1928; Ling, Liu, and Lim, 1928). The present observations do not furnish any evidence that the mitochondria take a direct part in cellular secretion. The only changes observed in the mitochondria were restricted to the zymogenic cells, and these were changes of shape and disposition. In the cells of the surface epithelia mitochondria are few in number, and many secretory granules are produced soon after the intake of food; it is unlikely, therefore, that morphological changes of the mitochondria were overlooked.

Intestinal Epithelium

The ease with which the intestinal epithelium may be stimulated to secretory activity has attracted many workers, and extensive observations have been carried out on active and inactive cells of invertebrate and vertebrate animals. Many of the earlier workers noted that the Golgi material and the mitochondria undergo morphological changes which are correlated with functional activity of the cells. Eklöf (1914) claims that he observed a large number of mitochondria which showed greater structural variations during the digestive process than after a fast of 24 hours. Miller (1922) maintains that in the final stage of secretion in rats mitochondria are absent from the intestinal epithelium, while Saito (1933) describes, in the same animals, a breaking up of filamentous forms into granules half an hour after feeding. Recently, Williams (1943) worked on the mitochondria of the intestinal cells of the Japanese salamander (*Triturus pyrrogaster*), and states that, during digestion and absorption as well as during fasting and inanition, all forms of mitochondria are present. He maintains that the correlation between the mitochondria and the stages of digestion is not merely one of shape, but of the relative number of the different forms present and of the distribution of the mitochondria in the cytoplasm. Accounts of the Golgi material of the intestinal epithelia are more numerous and more detailed. Corti (1926) and Liu (1930) are the only authors who claim to have observed the fragmentation of the Golgi material after feeding. Cramer and Ludford (1925), in their study of the role of the Golgi material during fat absorption in mice and rats, observed that the small amount of Golgi material which is present during the fasting condition swells up and enlarges after absorption of fat, so as to form a network which fills the area between the nucleus and the free border. They maintain that the absorption of food material, other than fat, is not associated with any changes in the morphology of the Golgi material. Weiner (1928), using various animals (frog, white mouse, field mouse, white rat, and axolotl), records changes in both the mitochondria and Golgi material during fat absorption. The Golgi material becomes more deeply impregnated with osmium tetroxide, and there is a thickening of the

threads of which it is composed. Subramaniam (1938), in his work on the Golgi material in the intestinal cells of *Lumbriconereis*, describes an increase in the number of Golgi grains during secretion, and an intimate relationship between the first secretory granules and the chromophobic region of the Golgi batonnettes. Jacobs (1929) makes a similar claim for the cells of the mid-gut of *Astacus leptodactylus.*

The present account shows that, apart from the morphological and structural differences characterizing a particular group of cells, there are marked differences in the manner of reaction of cells from different regions of the alimentary tract during fasting and feeding. The secretory cycle of the main cells is easily influenced and brought to the resting phase by a short fast; in the mucous goblet cells, however, secretory activity seems to be governed by entirely different factors. Besides this difference, the same kind of cells in various parts of the intestinal tract respond differently, and very probably perform different functions.

Gresson (1934), working on the mid-gut of *Periplaneta orientalis*, described two kinds of cells, i.e. secretory and absorptive; the former, which are filled with secretory granules, are predominant in the upper part of the mid-gut and are very seldom observed in the posterior part. This author maintains that undoubtedly the function of the posterior part of the mid-gut is mainly absorptive. Williams (1943), working on Japanese salamanders, noted that the response of the mitochondria to fasting and feeding is most pronounced in the anterior intestinal cells. During the present investigations it was observed that this is also true of the Golgi material, and that moving from the duodenum towards the posterior part of the alimentary tract the intensity of the changes of the Golgi material diminishes gradually and disappears completely in the lower parts (caecum, rectum). It was also noted that the main epithelial cells when brought to a resting phase by fasting become strongly sensitized to the subsequent administration of food and show prominent morphological changes. The present observations, following the routine of taking samples from the parts above and below the line marked by the descending bowel contents, show clearly that the first apparent secretory changes are initiated by the direct contact of the cell with food. That the cells present on the top of the villi respond more quickly than those situated below supports this statement. The gradual decline and total disappearance of the morphological changes in the posterior part of the intestine are no doubt connected with the various functions of the different parts of the intestinal lining. The present account agrees with previous contributions in that the proximal part of the intestine is chiefly concerned with secretion, and the distal part with absorption. These claims are supported by the following: Striking changes in the Golgi material of the upper part of the intestinal epithelium are visible as soon as the bowel contents reach this region, usually in less than half an hour after the intake of food. It is difficult to imagine that in such a short time digestion could take place to such an extent as to convert the food into an assimilable form, and consequently that the

changes in the Golgi are concerned with absorption. Secondly, the progressive phases in the production of the secretory granules clearly indicate that all these granules make their way towards the striated border of the cell. There is no indication that any of the granules take a different course, i.e. towards the basal part of the cell, as they would do if composed of absorbed material. Examination of material from birds killed at various times after fasting and feeding shows clearly that the Golgi material plays a specific part in secretion and that to deny its participation in secretory phenomena would be more than illogical.

Sudden and short-lived changes in the mitochondria during the first transitory phase, when the cell components seem to be mobilized for the secretory process, are an indication of their share in the synthesizing phenomena which terminate in the production of free granules.

The diminished morphological changes of all cell components in the lower parts of the intestinal tract undoubtedly indicate that secretion in this part (except mucus) must be very limited, or even non-existing, and that absorption does not actively influence the cell components.

SUMMARY

1. *Proventriculus*

The Golgi material of all the gastric cells, except the zymogenic cells, is situated between the nucleus and the lumen of the gland. In the zymogenic cells it always lies at the level of the bottom of the intercellular clefts. The apparent reversal of the polarity of the Golgi material of the zymogenic cells is due to the movement of the nucleus.

The mitochondria of the surface epithelium and the mucous neck cells are very delicate filaments. In the zymogenic cells thick rods and granules are usual. The functional stage is characterized by the presence of long rods with marked polar orientation.

Secretory granules arise in close association with the Golgi material of all the gastric cells. Feeding accelerates the evacuation of secretion and immediately stimulates new production. A total expulsion of secretory granules never takes place in any of the gastric cells.

2. *Gizzard*

The keratinoid material is a secretory product. The Golgi material does not undergo any changes after feeding. The secretory process is very slow and is independent of digestion. Secretion terminates with the degeneration and ultimate death of the cell.

3. Intestinal epithelium

The Golgi material of the epithelial cells lies above the nucleus. It shows marked changes of morphological and physico-chemical nature as soon as the resting cell is stimulated by direct contact with food.

The mitochondria are in the form of filaments, rods, and granules. Their polar arrangement is a constant feature. When the cell is first stimulated by food, the mitochondria stain very faintly.

Secretory granules arise in close association with the Golgi material; they move towards the glandular pole of the cell. Morphological changes of the Golgi material and mitochondria diminish and finally disappear in the posterior parts of the alimentary tract.

4. Goblet cells

The Golgi material lies above the nucleus. During secretory activity it enlarges greatly; it decreases in mass during the final stage of secretion.

The mitochondria are in the form of filaments, rods, and granules. With the accumulation of secretory material, the mitochondria collect on the border of the mucous mass and in the cytoplasm of the narrow part of the cell.

Secretory granules arise in close association with the Golgi material. The secretory process of these cells is autonomous and is not directly correlated with digestion.

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DESCRIPTION OF PLATE I (Photomicrographs ×1,200)

Fig. 15. Mucous neck cells; Golgi material in form of basket-like structure below the mucous mass.

Fig. 16. Zymogenic cells, showing Golgi material; transverse section of glandular tubules.

DESCRIPTION OF PLATE II (Photomicrographs ×1,200)

Fig. 13. Cells from a villus showing Golgi material; after 24 hours' fast.

Fig. 14. Cells from a villus upon direct contact with food; fragmentation of Golgi material.

Fig. 15. Cells from a villus 1 hour after feeding; Golgi material hypertrophied.
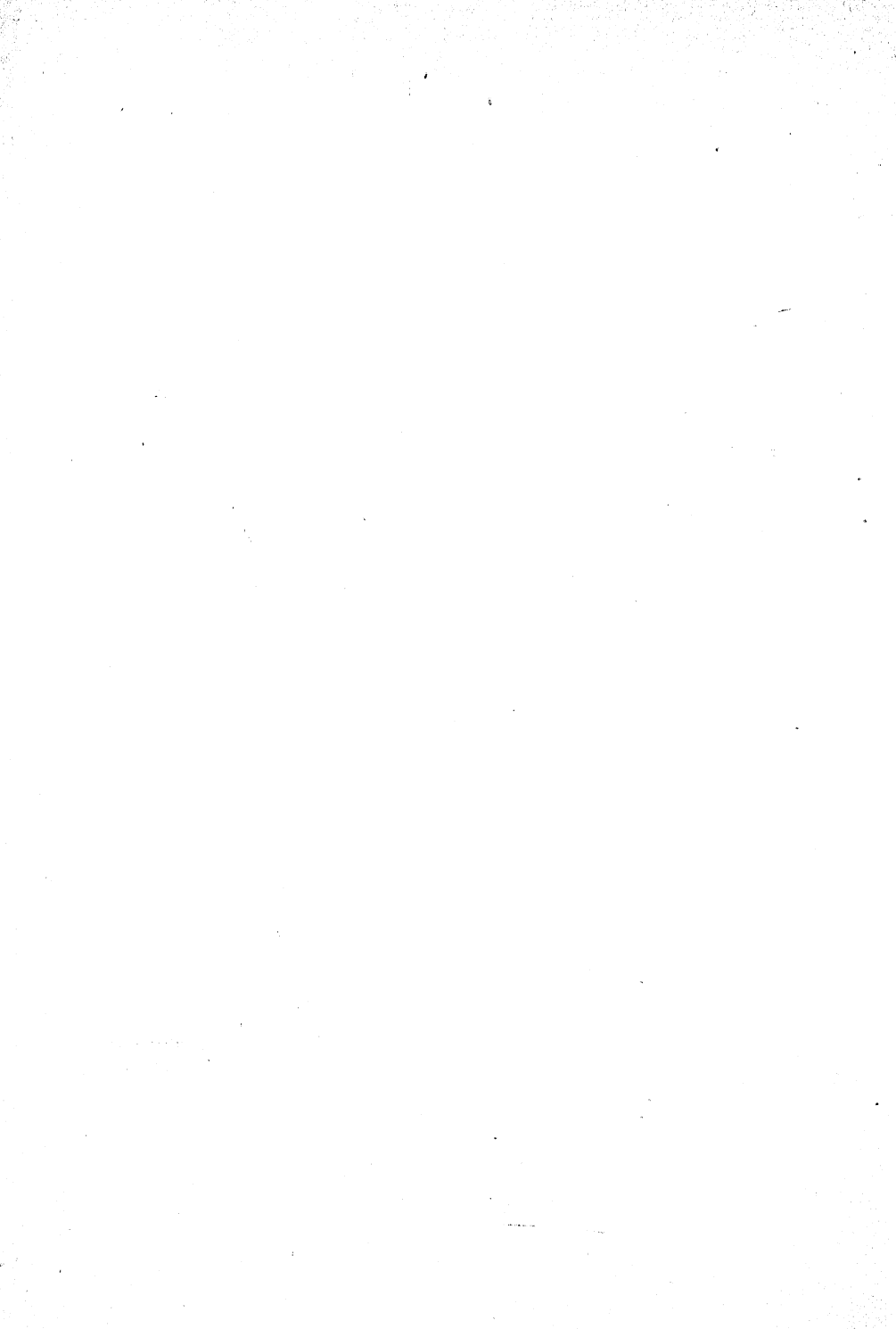
The Cytology of the Neurones of *Helix aspersa*

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With seven Text-figures and one Plate

WITHIN recent years the important investigations of Parat (1928), Hirsch (1939), Worley (1943), and Baker (1944) have extended our knowledge of the form, function, and cyclical activity of the intracellular organoids, more especially with regard to the so-called Golgi apparatus.

No longer is it permissible for the cytologist to rely solely on the appearance produced in cells by the classical methods of silver and osmium impregnation for his identification of these organoids. Such studies must be carefully controlled wherever possible by observation of living cells and by the application of vital and supravital staining. Worley (1946) in concluding his recent review of the Golgi apparatus emphasizes this point of view when he states: 'Studies of the Golgi apparatus should always involve a comparison between fixed and living tissue. No structure in the cytoplasm of either the vitally stained or the fixed cell should be accepted unless there is a fairly perfect correspondence between the two types of material. In general structures that blacken with prolonged osmication, and stain vitally with methylene blue may be considered a part of the total Golgi complement of the cell.'

In this study it was decided to carry out an investigation on living or freshly teased nerve-cells; first by direct observation with transmitted, dark field, and phase-contrast microscopy to identify as far as possible all the intracellular structures which could be identified on the basis of a specific and constantly recurring morphology; secondly to submit these structures to a series of procedures with vital dyes, histochemical tests, and further to record the effects of fixation, staining, and metallic impregnation of these bodies. Throughout the earlier part of this investigation no reference was made to the relevant literature with its host of conflicting views, since it was thought that it would have a baneful influence in forming preconceived and possibly ill-founded ideas of these structures. For this reason the familiar terms, Golgi apparatus, Golgi complex, mitochondria, &c., are not used within the section of this paper describing the observations but are reserved for the subsequent discussion.

After some thought the neurones of *Helix aspersa* were chosen for this study since this mollusc or closely allied species have almost a world-wide

distribution and the experiments described in this paper can be reproduced and verified easily by other investigators.

METHODS

The freshly teased nerve-cells of the post- and mesocerebrum of *Helix aspersa* were examined in a few drops of 0·7 per cent. sodium chloride containing 0·2 per cent. of 10 per cent. anhydrous calcium chloride. Transmitted, dark field, and phase-contract illumination were employed in this study. The phase-contrast microscope and equipment were identical with that used in a previous investigation of these cells (Thomas, 1947).

Supravital and intravital staining were carried out with neutral red chloride, methylene blue B.D.H. 5612137410131, nile blue, and Janus green B (Höchst) dissolved in the required concentrations in 0·7 per cent. sodium chloride solution containing 0·2 per cent. of 10 per cent. calcium chloride. For intravital staining about ½ c.c. of the solution was injected into the haemocoele with a fine hypodermic needle through the expanded foot of the snail at a point about midway between the head and posterior part of the metapodium. The animals were killed by quick decapitation while in the expanded condition.

For fixed material the following techniques were employed:

1. The acid haematein test of Baker (1946) followed by the pyridine extraction control test.
2. Altmann's acid fuchsine picric acid stain for mitochondria, following Schridde's fluid or Bensley's acetic, osmium, dichromate fixative.
3. The Azan stain of Heidenhain and Masson's technique following Zenker–Formol and Carnoy fixation.
4. Mann–Kopsch technique and the variant described on p. 455.
5. The sudan black B technique of Baker (1944) for the demonstration of the Golgi apparatus in frozen sections.

OBSERVATIONS

The Living Cell

The cerebral ganglia of *Helix aspersa* consist of two lateral masses connected by a supra-oesophageal commissure. Each lateral mass contains a fusion of three separate ganglia—a procerebrum consisting of small cells which according to Hanström (1928) are mainly association neurones, and the mesocerebrum and post-cerebrum. The mesocerebrum contains numerous large cells whose axons after a short course branch diversely. Some of these branches run via the central commissure to the ganglia of the opposite side, whereas the remaining fibres either pass to the pedal commissure or anastamose with the ipsilateral neurones of the post- and procerebrum. The post-cerebrum similarly contains large unipolar neurones which are concerned with tentacle reflexes.

These ganglia can be easily isolated by gentle teasing of the fused mass. Usually the cells of the post- or mesocerebrum were selected for study, as

their average size of about 50 μ in diameter makes them very suitable objects for high-power study with the microscope. Occasionally pieces of the small-celled procerebrum may by accident be included in the teased preparation, but with experience in manipulating the material suitable groups of medium-sized cells can be quickly selected and spread out between coverglass and slide.

Particular attention has been paid to the examination of the cytoplasm of these cells with both transmitted and phase-contrast illumination. In addition to the neurofibrils and the very small particles (the microneurosomes) pre-viously described (Thomas, 1947), two further categories (Text-figs. 1 and 2) of intracellular structures could be clearly discerned within these neurones:

1. Elliptical corpuscles with attached granules, hereafter described as spheroid complexes.
2. Minute filaments and coccoid chains.

The Spheroid Complexes

These structures or systems appear as a varying number of small refractile globules or spheroids scattered throughout the cytoplasm, each consisting of a spheroid of optically clear homogeneous material to which under optimal conditions of observation one or more still smaller dark granules can be seen to be attached. These dark granules occur irregularly around the periphery of the spheroid and are best seen by slightly reducing the illuminating pencil of light or by using phase-contrast microscopy.

When the cell is crushed between coverglass and slide so as to rupture the cell membrane these structures can be seen to flow out freely with the cytoplasm. On one occasion under these conditions the separation of one of the dark granules from its spheroid was witnessed and the impression was gained that the granule had a morphological identity of its own. It was henceforth decided to give the name spheroid complex to the combination of the colourless spheroid and its accompanying granule or granules. It must be mentioned that the term spheroid complex at this stage is provisional and does not mean that the attached granules or granule can always be demonstrated, since in some cases they may be situated on the deep aspect of the spheroid and (being turned away from the view of the observer) will therefore be invisible.

On the average the spheroids are about 1·5 μ in diameter, but they vary quite considerably in size within any one cell (Text-fig. 1). The attached granules are too small for accurate measurement, but they are considerably larger than the individual microneurosomes. In general these complexes tend to be aggregated in the cytoplasm surrounding the nucleus. Occasionally in the larger nerve-cells large spheroid complexes occur which present a some-what scalloped outline, resembling a diminutive mulberry (Text-figs. 1, 2, 5).

These mulberry forms are sometimes present in very great numbers within the neurones of fully grown specimens of *Helix* and tend to be accumulated at one pole of the cell. Such a mass may be clearly visible even

with the low powers of the microscope, since each mulberry spheroid being tinged somewhat yellow gives to the whole group a distinct golden-yellow colour.

The Filaments and Coccoid Chains

In addition to the spheroid complexes a number of slender colourless filaments, usually somewhat slightly bent towards one end, are scattered
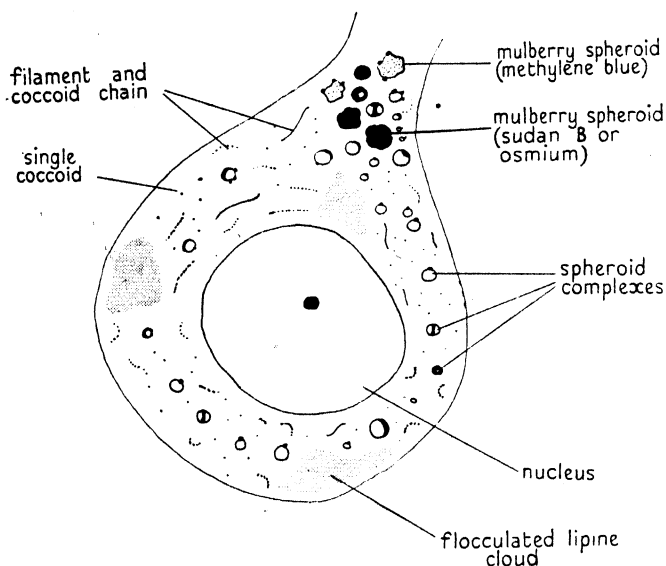
TEXT-FIG. I. A composite diagrammatic drawing of a neurone of *Helix aspersa* showing the morphological characteristics of the two categories of intracellular organoids observed with the techniques used in this study.

throughout the cytoplasm. Further, there may occur tiny chains of 5 or 6 small refractile bead-like structures, resembling somewhat in appearance and size a chain of streptococci (Text-fig. 1). It is characteristic of both these filaments and chains that they are scattered about in a haphazard fashion and are never collected into regular rows like the neurofibrils. A number of discrete single coccoids of the same order of size as the coccoids of the chain forms described above can also be seen within the cell and, in fact, predominate if the filaments or chains are absent.

Vitally Stained Material

Neutral red chloride. This dye has the power of staining strongly the spheroid complexes to the exclusion of all other structures within the cell. Concentrations of dye as low as 1:100,000 were used supravitally, and

although at such a dilution no colour could be detected in the surrounding medium, the cells after a period of 20–30 minutes imbibed the dye, concentrating it very selectively within the spheroid complexes, revealing them as bright brick-red structures.

When neutral red in saline is injected into the haemocoele of the snail and the animal killed within 10–15 minutes, the small dark granules of the spheroid complexes are in addition especially well revealed as very strongly stained granules, whereas the related spheroid itself has under the conditions of the experiment only assumed a slightly reddish tinge. Longer applications of the dye by the injection route will produce an exactly similar appearance to that of the supravital method, but the differential coloration of the complex will be lost. Likewise preparations that are showing full and generalized staining of the complex can be made to reproduce the spheroid plus granule appearance by simply waiting for some minutes longer for the dye to begin to be reduced to the leucobase under the relatively anaerobic conditions obtaining beneath the coverglass. As the red colour fades from the spheroid itself the attached granule or granules are beautifully revealed with the utmost distinction, and will remain as much brighter red granules resisting tenaciously further reduction of the contained dye. In other words, during progressive vital coloration the granule always stains before the spheroid, but later, after the whole system is homogeneously coloured red, the granule then becomes invisible and only finally reappears as reduction to the leucobase produces differentiation of the spheroid complex into its constituent parts.

Methylene blue 1:10,000–1:100,000, like neutral red, produces best results when it is injected in saline solution into the haemocoele. Staining is similarly restricted to the spheroid complexes. The attached granules are particularly well shown. When the dye is allowed to act for short periods of 10–30 minutes, most of the spheroids are of a faint pale-blue colour and their attached granules are by comparison very sharply shown as dark blue. If the dye acts for an hour or more the dark staining of the granule is lost, but stained areas of varying pattern appear to be closely applied to the spheroid. A selection of the various shapes these stained structures may take is represented in Text-fig. 2. This drawing was made during observation of a group of living cells and represents the forms more frequently seen. These darkly staining areas appear as complete or local thickenings in what is usually a pale-staining investment to the spheroid. This investment when stained completely and seen in optical section appears as a rim. In other cases there is an appearance of a crescent which is perhaps due to partial staining of this rim or to staining of a locally thickened area of the rim. In still other cases, columns or complete bands appear around the periphery of the spheroid and these again may be due to viewing the locally stained crescentic areas from the side.

It might be assumed that these appearances of a complete or incompletely stained investment were due to a swelling and elaboration of the simple granule. This cannot be definitely established since in a few cases spheroids

possessing rims or localized areas of darkly staining material had distinct granules attached to other parts of their circumference (Text-fig. 2).

After prolonged application of the dye, the material of the spheroid itself

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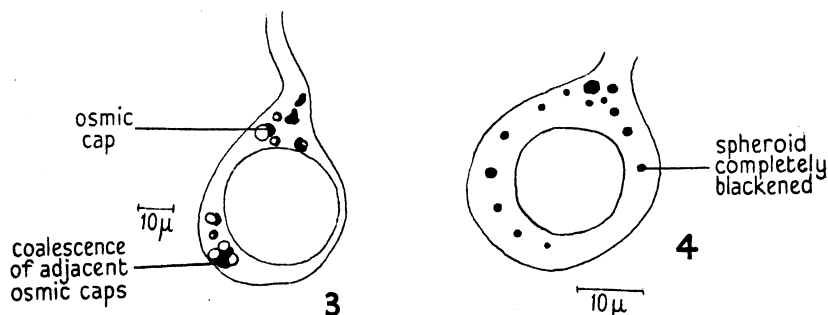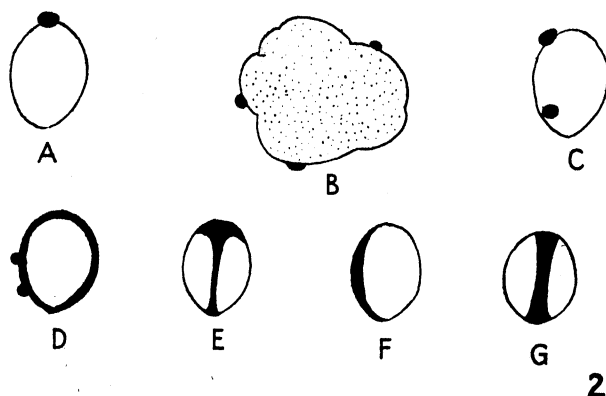
TEXT-FIG. 2. The various shapes of dark-staining granules (A and C), rims (D), crescents (F), and bands (E and G), taken by the chromophil substance when the spheroids are stained with vital methylene blue. B represents a mulberry spheroid with attached granules of chromophil substance. The chromophobe area has accumulated a mass of yellowish Golgi product (the lipochrome of Legendre). With methylene blue this product is but faintly stained, but it is itself coloured with sudan or osmium. Cf. Text-figs. 4 and 5.

TEXT-FIG. 3. A camera lucida drawing of a smeared neurone subjected to warmed osmic vapour. The chromophobe spheroids exhibit exaggerated caps or 'cocked hats'. Coalescence of adjacent caps to form an irregular black lump is illustrated.

TEXT-FIG. 4. A camera lucida drawing of a neurone completely immersed in a solution of osmium tetroxide. The spheroids and mulberry forms are completely blackened. No differentiation into chromophil and chromophobe areas are visible.

in most cases appears to be stained homogeneously throughout. Finally, coincidently with death of the cell, the nucleus and cytoplasm become stained.

Nile blue 1 : 10,000 in saline will give precisely the same results as methylene blue. The attached granules are stained blue while in some cases the substance of the larger spheroids is tinged a reddish colour.

Janus green B (Höchst) 1:10,000 was applied by the supravital method. Considerable difficulty was experienced in getting the stain to work and in successful preparations only a few cells took up the dye. This substance was found to colour the delicate filaments and coccoid chains and individual coccoids, and to leave the spheroid complexes unstained. However, when once staining has occurred the great specificity of this dye is dramatically exhibited. Best results were obtained by compressing a group of cells and then examining those at some distance from the edge of the flattened mass. Cells at the edge usually show diffuse staining of the nucleus and cytoplasm, whereas cells in the centre are wholly unstained. Somewhere along this gradient optimal conditions obtain for the reaction and preparations must be very carefully searched for successful results before being discarded as useless.

Osmium Tetroxide

Freshly teased cells were spread out on a coverglass and then inverted over a cavity slide containing a drop of 2 per cent. osmium tetroxide. Cells at the periphery of the preparation became immersed in the solution, whereas the more centrally placed cells were separated from contact with the osmium tetroxide by a small air space, and hence were only subjected to the action of the vapour. As a result the action of this reagent could be watched progressively under two sets of conditions. In addition some preparations were placed in an incubator at 37° C. and inspected every few minutes in order to observe the effect of heat upon the osmication process.

Cells subjected to the action of the vapour only showed a progressive blackening of the cortex of the spheroid complex. This blackening first appears in optical section as a delicate complete rim, but very soon one portion of the periphery of this rim becomes appreciably thickened to form first a crescent-shaped cap which is finally expanded into a much larger structure resembling in relation to the almost unblackened central core a miniature cocked hat. Text-fig. 3 gives a good representation of these expanded crescents produced by prolonged osmication. Usually the base of the cap or cocked hat of blackened material is continuous with the faint blackened rim to the whole spheroid. The blackened tract of material then resembles a signet ring placed over a ball (the spheroid).

When heat is applied osmicated caps of adjacent spheroids not infrequently are seen to coalesce to form an irregular black lump (Text-fig. 3). The actual process of fusion can, in fact, be witnessed. As coalescence occurs in those cells which were subjected to the heated osmic vapour and not in cells immersed in the cool fluid, it is probable that the heat produces some desiccation of the preparation as a necessary preliminary condition for producing this effect. Coalescence of the osmicated caps of adjacent spheroids was not witnessed in material kept at room temperature.

In cells wholly immersed in a solution of osmium tetroxide the spheroids do not show attached caps or 'cocked hats': instead there is a gradual and

progressive blackening of the whole spheroid. At all stages the spheroids appear to be quite homogeneous without differentiation into an outer blackened and inner clear zone as is the case with osmic vapour.

Fixed and Stained Preparations

The formal-sudan-black technique of Baker (1944) produces in the cell a picture somewhat intermediate between that of osmic vapour and that of direct immersion in osmium tetroxide solution (Text-fig. 5). Most of the large mulberry spheroids are totally blackened with the sudan, but in addition all degrees of the differentiation of the spheroids into an outer blackened and a pale inner zone occur—signet-ring forms with complete rims and thickened caps, incomplete rims appearing as crescents and sometimes only. one or more discrete granules attached to a colourless spheroid. In a large cell the total number of these systems or complexes may be very great indeed, and the casual observer may mistakenly dismiss the smallest complexes as a collection of granules. However, careful study will show that invariably the 'granule' is, in fact, a compound structure. The tiny coccoid rods and filaments stainable with Janus green do not blacken with this technique and remain invisible in these preparations, but sometimes one finds a ground-work of extremely fine black granules forming a dark zone surrounding the nucleus, especially in the larger nerve-cells. These dark areas can only be adequately described as perinuclear clouds, for the precise resolution of their individual particles is a matter of the utmost difficulty. In some cases this material may appear in flocculent, more or less rounded patches occurring throughout the cytoplasm.

The acid haematein test for lipines after Baker (1946) was applied to this material and the observations controlled by the pyridine extraction test. Baker's technique stains the spheroids a bluish-grey colour. When observed in optical section the impression is gained that the lipines form an outer skin to the spheroid which itself does not give a positive reaction with the acid haematein. In some cells a diffuse cloud of lipine occurs throughout the cytoplasm (Text-fig. 6). In their general morphological distribution these lipine clouds correspond to the diffuse sudanophil material observed with the sudan black technique of Baker (1944). With the pyridine extraction test the cells show a clear cytoplasm with distinct staining nuclei. The spheroid skins and diffuse clouds observed with the acid haematein test are not visible. In some cells complete 'negatives' or 'ghosts' of the spheroids can be seen as clear spaces in the cytoplasm.

With the Mann–Kopsch technique a very different picture of the cell is obtained from that observed in the vital studies and with the techniques described above.

Instead of a number of spheroids with attached rims, crescents, or granules, the cytoplasm is crowded with a large number of osmiophilic curved rods or batonnettes resembling the batonnette of the molluscan 'Nebenkern'. Each rod is surrounded by a more or less osmiophilic zone of cytoplasm. This

'neoformation' within the cell is at first sight very puzzling, and it was thought that these curved rods might be the rims or crescents of the spheroids distorted somewhat by the fixation and thickened by aggregation of osmium
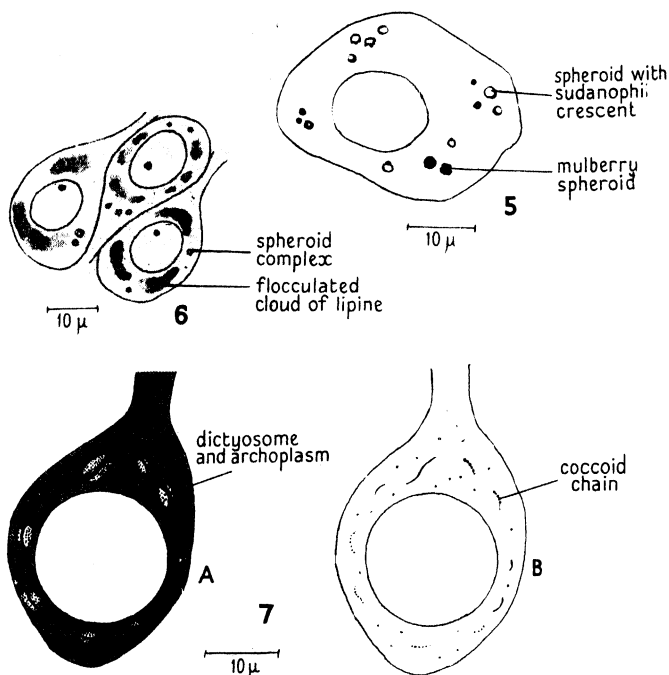
TEXT-FIG. 5. Camera lucida drawing of a neurone prepared by the Baker technique. A close representation of the living and vitally stained cell is obtained. The mulberry spheroids are totally blackened owing to the fatty Golgi product contained within the spheroids colouring with the sudan black.

TEXT-FIG. 6. A group of neurones prepared by the Baker acid haematein test. Flocculated clouds of lipine occur throughout the cell. The spheroids are visible because their skins give a positive reaction for lipine.

TEXT-FIG. 7 A. A neurone prepared by the Mann–Kopsch technique. The so-called Golgi dictyosome and archoplasm can be readily seen, produced by the use of this method. B. The same cell after partial bleaching with the Verrati bleacher. The 'archoplasm' disappears and the dictyosome, greatly reduced in thickness, can usually be resolved into a row of coccoids. This evidence supports the view that the dictyosomes are artifacts produced by the over-impregnation of the mitochondria, and do not represent the true Golgi apparatus of *Helix*.

particles. Further, it was possible that the spheroid itself might have collapsed and dispersed to form the lighter osmiophilic zone around the batonnettes.

This idea was put to the test by first locating a suitable group of cells from a Mann–Kopsch preparation, tracing with the camera lucida an accurate outline of the cells, and marking within the exact position of some of the most distinct batonnettes. The coverglass was then soaked off the slide and the

preparation passed through xylene and the alcohols to water. The osmium was then completely removed from the sections by immersion in the acid permanganate bleacher of Verrati (see Gatenby, 1939). The slide was then washed in water, placed in 70 per cent. alcohol, and then treated with a saturated solution of sudan black B in 70 per cent. alcohol for 10 minutes. After a quick rinse in 50 per cent. alcohol it was washed in water and mounted in Farrants's medium.

The same cells were then brought into the field of view and a fresh drawing made of the appearances produced by the application of sudan black. By comparing the drawings it was possible to assess the results of the two different techniques upon the same cells.

The 'restained' cells showed very precisely numbers of typical spheroid complexes which were previously invisible in the same cells after the Mann–Kopsch technique. It was obvious that the mercuric osmium fixative had preserved the spheroids but the subsequent osmication had failed to exhibit them. Further, the dehydration, embedding, and bleaching procedures had failed to remove them from the cells. In fact the bleached and sudan-treated sections compared more than favourably with the frozen section material prepared by the original Baker technique.

In this way it was established beyond doubt that the characteristic batonnette obtained by the Mann–Kopsch technique does not bear any positional or morphological relationship to the spheroid complexes exhibited by the Baker technique. Further, as these sudanophil spheroids can be definitely correlated with the spheroids seen in the living cell and the vitally stained preparations, whereas the osmiophilic batonnette did not appear to have such a counterpart, it was thought possible that perhaps the batonnettes were either artifacts produced by non-specific reduction of osmium tetroxide or perhaps a structural component of the cell which had hitherto resisted all attempts to disclose it.

This problem was attacked by arranging for a number of 'successful' Mann–Kopsch preparations exhibiting intracellular batonnettes to be bleached in the Verrati bleacher for graded periods of time so as to reproduce in a series the various appearances of the batonnettes as the osmium was regressively removed. Experimentally this regressive technique is a much easier procedure than to arrange for a series of progressive impregnations of the batonnette.

The results obtained were very illuminating. The first part of the reduced osmium to disappear during the bleaching process is the brownish zone surrounding the batonnette. This is followed by the gradual removal of osmium from the rod itself which consequently becomes much thinner in appearance. Not infrequently some of the batonnettes are reduced to a row of distinct cocci. These stages are illustrated in Text-fig. 7. Finally, if the bleaching is continued all the osmium can be reduced from the sections.

The absence of any impregnation of the spheroids by the Mann–Kopsch technique was somewhat unexpected, as these bodies had already been impregnated successfully by osmic vapour and with immersion in simple 2 per

cent. osmium tetroxide. The combination of mercuric chloride with osmium tetroxide in the Mann–Kopsch fixing fluid was considered to be responsible for the shifting of the selectivity of the osmium from the spheroids to the counterparts of batonnettes in the living cell. A series of experiments was planned with variants of the Mann–Kopsch technique, in which the fixative action of the sublimate was dissociated from the subsequent impregnating action of the osmium tetroxide. The following variant proved the most reliable for impregnating the spheroids with osmium:

1. Fix whole ganglia in a saturated solution of mercuric chloride in 0·75 per cent. sodium chloride.
2. Remove excess of mercuric chloride by thorough washing in running water for 12 hours.
3. Leave in distilled water overnight.
4. Transfer to a tube containing enough of a simple 2 per cent. solution of osmium tetroxide to cover the tissue, close the tube with a rubber stopper, and leave in the dark at room temperature for 1–6 days.
5. Pieces should be removed at the end of each day and washed for 3 hours in running water.
6. Dehydrate in alcohols, embed, and cut.

With this method the complete spheroids are thoroughly impregnated and appear as a number of homogeneous black globules.

Differentiation of the spheroid complex into darker rims or caps attached to lighter more translucent spheroid bodies is not evident. If these preparations are now regressively bleached with the Verrati bleacher the impregnation can be shown to extend evenly throughout the whole mass of the spheroid. As the result of bleaching a clear rim appears at the periphery of the black globule and this increases *pari passu* with the reduction in its size until finally a completely decolorized negative or 'ghost' of itself is produced. Such a chain of events can only be consistent with the progressive removal of the outer layers of a homogeneously blackened spheroid.

Similarly it was found that the spheroids can be impregnated, although rather faintly, during the course of fixation and without subsequent post-osmication if tissues are placed in Schridde or Bensley's acetic osmium dichromate fixative. The blackish spheroids are at once visible on removing the paraffin from the sections and before staining. Some sections exhibiting this phenomenon were stained by the technique of Altmann with acid fuchsine and differentiated in aqueous picric acid.

A number of the lightly osmicated spheroids prepared by this method were seen to possess a clearly differentiated bright fuchsinophil rim which in some cases showed local thickening, giving to the whole a signet-ring appearance exactly comparable with the similar appearances produced by osmic vapour.

The Altmann technique also reveals the presence of slender rods and coccoid chains together with individual cocci. These structures are morpho-

logically identifiable with the filaments and chains exhibited with Janus green B used as an intravital stain on living cells.

The staining reactions to acid fuchsine of the two distinct categories of intracellular bodies, the attached rims or caps of the spheroid complexes and the detached and separate coccoid chains or filaments, thus appear to be identical.

DISCUSSION

It now remains for us to review the various categories of intracellular structures described above in the light of previous investigations upon the invertebrate nerve-cell, and to endeavour to correlate the particular structures seen within the living neurone with the appearances produced in these cells by application of the technical procedures of fixation, staining, and metallic impregnation. Four distinct and apparently unrelated definitive cellular components have been described by other authors as occurring in these cells:

1. The Golgi apparatus.
2. Mitochondria.
3. Metaplasmic granules (lipochrome of Legendre, neuro-secretion granules of Scharrer, &c.).
4. Nissl substance or tigroid bodies.

The Golgi Apparatus

A number of workers such as Legendre (1909), Kolatchev (1916), Brambell (1923), Brambell and Gatenby (1923), and Boyle (1937) have described the Golgi body or dictyosome of the molluscan nerve-cell as consisting of a somewhat curved osmiophil batonnette associated with a more or less chromophobe 'archoplasm'. Bodies answering to this description can be readily seen following the Mann–Kopsch technique. In order to prove whether or not these appearances were due to partly impregnated spheroids or perhaps spheroids distorted during fixation, Mann–Kopsch preparations were bleached and recoloured with sudan black. The results of this experiment are illustrated in Plate 1. A photograph (upper figure) was first taken of the dictyosomes, and another after bleaching and restaining the preparation (lower figure). By comparing the two appearances of the same cells it can only be concluded that the spheroids (which can be seen in the living cell) are entirely different bodies from the batonnettes and do not in any way contribute to their production in the fixed cell.

Recently Worley and Worley (1943/4) and Worley (1946) in a series of interesting papers have given very strong support to the view of Hirsch (1939) and others that the true Golgi apparatus consists of a complex composed of chromophil and chromophobe portions. They base this differentiation on the reaction of the apparatus during the life of the cell to a dilute solution of methylene blue. Worley (1946) stressed, however, that the methylene-blue-staining bodies must also blacken with prolonged osmication before conclusive proof of their being part of the total Golgi complement of the cell

dictyosome
and
archoplasm
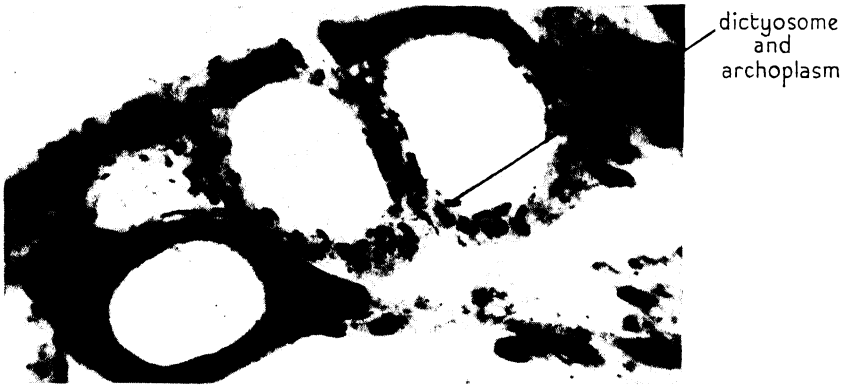
Photomicrograph of a Mann–Kopsch preparation showing a typical rod-like
dictyosome embedded in a mass of archoplasm.

spheroid

The same preparation following bleaching with the Verrati bleacher and recoloured with
the Baker sudan black technique for the Golgi apparatus. The identical cells are rephoto-
graphed. The dictyosomes and archoplasm are lost or the dictyosomes are reduced to fine
mitochondria-like threads or coccoid chains. A spheroid complex showing differentiation
into chromophil part and chromophobe centre is shown. It should be noticed that this
complex remains invisible in the Mann–Kopsch method.

THOMAS.—PLATE I

is obtained. Baker (1944) gives good reasons for believing that the methods used to produce the classical Golgi network cannot be relied upon to give an accurate picture of the structure of the Golgi element during life. Using sudan black B, a colouring agent with an intense affinity for lipoids, he has developed a new technique for showing the Golgi element which has given valuable results on a number of diverse cells, including the cells of the anterior mesenteric ganglion of the rabbit. In these vertebrate nerve-cells Baker describes the Golgi apparatus as consisting of a number of separate vacuoles each surrounded by 'an intensely sudanophil skin which is not uniformly thick all over'. In optical section this skin appears as a ring or crescent enclosing a colourless centre or vacuole. These compound bodies closely agree with the chromophil and chromophobe complexes of Worley and Hirsch.

Young (1932) in cephalopod material observed numerous neutral-red-staining spheres, with attached methylene-blue-staining granules scattered throughout the cytoplasm of the nerve-cells. He believed that similar methylene-blue-staining and osmiophil granules in close proximity to the dispersed spheres probably represented the Golgi apparatus. He could find no evidence of either a Golgi batonnette or a classical network within his material.

Covell and Scott (1928) and Murray and Stout (1947) working on the vertebrate nerve-cell found a close correlation between the neutral-red-staining and osmiophil granules and identified these bodies as a dispersed Golgi apparatus.

Ever since the pioneering work of Parat (1928) we have been entering a new period of cytological inquiry wherein the old criteria for the identification of the Golgi apparatus based on the use of silver and osmium impregnation techniques without adequate control by vital studies must be seriously questioned.

The earlier work on the molluscan 'Nebenkern' and the questionable identification of its contained rod-shaped and easily visible lepidosomes as the Golgi body of the spermatocyte has undoubtedly had its effect upon all subsequent work on invertebrate nerve-cell cytology. Workers have only used those variations of metallic impregnation techniques that successfully produce in the cell a lepidosome-like structure. Slides which show an impregnated curved rod or batonnette have been hailed as 'good' preparations of the Golgi apparatus, and slides not showing this appearance have been arbitrarily rejected as failures. From my own observations I have come to reject the hypothesis that the batonnette and its attached 'archoplasm' represents any part of the Golgi body of *Helix* neurones.

On the contrary, the invertebrate nerve-cell has been shown to contain during life a number of discrete spheroid bodies possessing an outer skin of a sudanophil, osmiophil, and methylene-blue-staining lipoid containing a relatively chromophobe but neutral-red-staining core. A yellowish, fat-solvent-soluble and relatively less osmiophilic material gradually accumulates within

the centre of the spheroids to produce a mulberry-shaped mass, which may, however, retain some trace of the external skin attached to it. I identify the spheroid bodies as the true Golgi complement of *Helix* neurones, and the yellowish material as Golgi product.

The Mitochondria

Few workers would object to the classification of the structures stainable during the life of the cell by very dilute Janus green B as the mitochondria. These slender filaments or coccoid chains are the only morphological counterparts, visible in the living cell, of the batonnettes of earlier workers. The resemblance becomes strikingly apparent if the batonnettes are only partly bleached by the Verrati bleacher, whereby they are greatly reduced in thickness, and usually appear as a moniliform chain of coccoid particles (Text-fig. 7). Kolatchev himself (1916) observed that the mitochondria of *Helix* were linked to the batonnettes through a complete chain of intermediate forms, and he advanced the hypothesis that the batonnettes were derived from the mitochondria. Under the conditions of the Mann–Kopsch technique the slender filaments and coccoid chains are thickened by the deposition of successive layers of reduced osmium, which produces within the cell an apparently *de novo* structure, the batonnette. In most Mann–Kopsch preparations, besides the fully formed batonnettes, numbers of darkly impregnated smaller granules of various shapes and sizes occur. It is difficult to say exactly what these granules are, but possibly some of them are impregnated individual coccoid mitochondria.

The cytoplasm also contains numerous irregularly shaped flocculated masses, slightly impregnated by osmium and appearing light brown in colour. These masses can be seen after a wide variety of fixations and stains, and their occurrence in the Mann–Kopsch material and their frequent association with the batonnettes is not surprising. These flocculated masses when associated with the batonnettes are described as 'archoplasm' by some workers, but it is not at all uncommon to see many such masses, unaccompanied, in between the batonnettes and not attached to them.

Boyle (1937), on the basis of their reaction to the dye nile blue, concluded that the spheroids were 'metamorphosing mitochondria' in the process of swelling up to form lipoidal globules.

Mr. A. J. Cain (1947) of this Department, who has recently published a critical survey of the histochemical uses of nile blue, informs me that when this dye is used at the concentration employed by Boyle (1:10,000 in 0·75 per cent. NaCl) a blue reaction given by cellular granules cannot be interpreted as indicating the presence of lipoids. Under these conditions the dye will react simply as a basic dye and in my own experience it will give results very similar to methylene blue, staining the chromophil portion of the Golgi system only.

Blue staining with nile blue is completely unspecific (Lison, 1936): it can only be used as a basis for histochemical inferences if the body concerned is

already known (through the use of sudan black or other colourants) to be lipoidal, in which case a blue colour does indicate the presence of lipines or fatty acids or both.

The Metaplasmic Granules

Legendre (1909) was one of the first investigators to describe the presence of granules of lipochrome within the nerve-cells of *Helix*. Gatenby (1923) and Gatenby and Hill (1934) confirmed this finding of Legendre but differed from Parat, claiming that they are stainable with neutral red but not with osmium tetroxide.

The term lipochrome is certainly a very ill-defined one from a histo-chemical standpoint. Gatenby thought that the granules might possibly be composed of lecithin.

I identify the lipochrome granules of Legendre and the lecithin granules of Gatenby with the mulberry forms of the spheroids (Text-figs. 1, 2, and 5). These forms are linked by a perfect series down to the smallest spheroid complexes, and studies with vital methylene blue indicate clearly that even the largest of these yellowish granules have attached to them a few granules of the chromophil portion of the Golgi apparatus. It seems clear that this yellowish material is formed within the chromophobe portion of the spheroids and finally, as the amount increases, it distorts the spheroid into an irregular mulberry mass. This transformation is probably associated with the rupturing or thinning of the outer chromophil envelope, which then becomes a discontinuous investment only retaining contact with the mass at a few parts of its circumference.

The methylene-blue-staining granules attached to the spheroids in *Helix aspersa* seem to correspond to those described by Young (1932) in cephalopod material. Young, however, observed aggregations of similar granules which he considered to be the Golgi apparatus completely free, grouped about the spheroids and unattached to them. I have not met with this appearance within living and vitally stained cells of *Helix aspersa*, but when ganglia are fixed and then stained either with iron haematoxylin, Azan, or Masson's technique groups of bright-staining granules, usually fuchsinophil, but sometimes staining with either the light green or the aniline blue, do occur grouped around characteristic clear spaces which are left in the cytoplasm as the ghosts of the spheroids when their lipoidal content has been dissolved in alcohols or xylene, previous to embedding. These bright-staining granules may be produced by fixation and staining of an external or 'extrasomal' Golgi product as has been described in *Mytilus* by Worley (1944).

Occasionally, too, masses of a clear pale-staining colloid are seen within fixed nerve-cells of *Helix*. Morphologically these fuchsinophil or iron-haematoxylin-staining granules and colloid masses are identical with those shown by Scharrer and Scharrer (1940) to occur within the nerve-cells of a number of invertebrates as well as vertebrates and interpreted by their school of workers as a secretory antecedent or precursor of a neurohormone.

The methylene-blue granules of the spheroid complexes appear to merge into the rims or crescents about the spheroids as staining progresses and it is possible that they represent a zone of maximal dye-concentrating power within the chromophil substances; for they are always the first to appear during progressive staining of the chromophil substance and the last to bleach when reduction of the dye to its leucobase commences.

The outer sheath or skin of the spheroid complexes when completely stained with methylene blue usually shows a striking similarity to the appearances produced by sudan black and osmium tetroxide vapour preparations, so much so that all doubt as to the rims, crescents, or caps exhibited by these methods being one and the same structure ceases to exist.

The central portions of the spheroids stain vitally with neutral red, irrespective of the presence of this endosomal product, and by definition comprise the vacuome of Parat. This author failed to recognize that this neutral-red-staining material is itself contained within a lipoidal capsule. From the researches of Duthie (1933), Hirsch (1939), and Worley (1946) it seems almost certain that the chromophobe spheroid is the main site for the collection of Golgi product (the endosomal product of Worley). In *Helix* the substance of the spheroids will occasionally stain a pink colour with nile blue at 1 : 10,000 in saline, and in addition it is possible to blacken it throughout its substance with osmium tetroxide according to the technique described on p. 455 of this paper. However, although the Golgi product itself seems on this evidence to be possibly lipoidal, it is certain that a limiting membrane consisting of lipines, and stainable with acid fuchsine after dichromate mordanting, can be very clearly shown to exist as a separate morphological component. This limiting membrane can only be identified with the chromophil zone of Worley, the Golgi externum of Hirsch, and the sudanophil dense lipoid-containing substance of Baker.

The Nissl Substance

No aggregations of material resembling the classical Nissl clumps of the fixed cell can be seen within living cells either by ordinary or phase-contrast microscopy.

In fixed mollusc tissue the Nissl substance has been studied by various workers. Garaieff (1909), Kunze (1921), Brambell and Gatenby (1923), Hanström (1928), and Boyle (1937) found the substance to consist of discrete granules distributed evenly throughout the cytoplasm. Erhard (1912) and Young (1932) with cephalopod material describe a homogeneous mass lying in the outer part of the cell.

My own findings indicate that this substance is most probably formed by coagulation of small discrete granules perhaps identical with the microneurosomes previously described in *Helix* (Thomas, 1947). Individual microneurosomes can be stained with both neutral red and Janus green, but they cannot be seen within the living cell unless the cell is slightly crushed by pressure of the coverglass. Apparently they are held within an optically

similar gel during life, but with pressure the physical nature of the medium in which they are embedded changes to a fluid consistency in which the individual particles can move about freely. A concomitant slight alteration in the refractive index of the surrounding fluid enables the granules with their intense Brownian movement to be observed.

Actively moving microneurosomes can be coagulated on the slide by running fixing fluids under the coverglass, and the resulting globule of cytoplasm can be shown to stain strongly with the basic dyes usually employed to stain the Nissl substance.

The hypothesis that the microneurosomes are the physical basis for the Nissl substance would account for the observed fact that typical 'grumes' of the tigroid body cannot be seen within the living cell, and it would further account for the various forms this material may take in the same cell following different fixatives.

In conclusion I wish to thank Dr. J. R. Baker for his helpful advice and criticism throughout this study, and for taking the photomicrographs of Plate 1; also to Professor A. C. Hardy, F.R.S., for his kindness in providing me with such excellent accommodation within his department.

SUMMARY

1. Direct observation of the living nerve-cells of *Helix aspersa* reveals the presence of two categories of cellular organoids:

(a) Spheroid complexes consisting of a central core of chromophobe material covered wholly or in part by a chromophil lipine substance.

(b) Filaments and coccoid chains, and dispersed coccoid particles.

2. Evidence is put forward identifying the spheroid complexes as a dispersed type of Golgi apparatus and the filaments and coccoids as the mitochondria.

3. The chromophobe core of the spheroids is identified as the vacuome of Parat and is the site for the formation of an endosomal Golgi product (the lipochrome granules of earlier workers).

4. The so-called Golgi dictyosome and archoplasm cannot be observed in living cells and are probably an artifact of technique produced by an over-impregnation of the mitochondria with silver or osmium.

The drawings have been made with the assistance of a camera lucida. The photomicrographs were taken by Dr. J. R. Baker with a technique described by him in a special article on photomicrography in the *Journal of the Royal Microscopical Society*, vol. 62, p. 112. He used a Reichert 2 mm. fluorite immersion lens and Watson 14 Holos eyepiece.

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Further Remarks on the Histochemical Recognition of Lipine

BY

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With one Text-figure

IN an earlier paper (Baker, 1946), a technique was described for the histochemical recognition of lipine. This technique was called the acid-haematein test. It was shown that lipines, after appropriate fixation and subsequent mordanting with potassium dichromate, display a special affinity for haematein, with which they react to give a blue, blue-black, or grey colour. If certain precautions are taken, lipines can thus be distinguished from other substances occurring in the tissues of plants and animals. It was found, however, that the crude galactolipine used in the investigation reacted much less strongly than lecithin, cephalin, and sphingomyelin. It will be recollected that galactolipine differs from these other lipines in containing no phosphoric acid radicle. The suggestion was made that if the galactolipine used in the test had been completely free from contamination with sphingomyelin, it might have proved negative to the test. If so, the acid-haematein test would be a test not for lipines in general, but for lipines other than galactolipines; that is to say, it would be a test for phospholipines. It is the purpose of the present paper to report the reaction of purified galactolipine.

In the earlier work, the lipines were all prepared by Weil's (1930) method. In the present investigation, crude galactolipine was prepared from the brains of sheep in the same way, but it was then purified according to the method of Rosenheim (1913). It was deposited on cigarette-paper, as before, and subjected to the acid-haematein test. The result was negative, for the colour given was not blue, blue-black, or grey, but yellow, tinged with greenish-brown. Other lipines, subjected simultaneously to the test as controls, gave positive reactions. The colour given by the galactolipine developed to a considerable extent in the hot potassium dichromate solution, before treatment with the dye began.

It was thought desirable to study the reactions of small globules of the various lipines to the acid-haematein test. To achieve this, each lipine was mixed with a thick sol of celloidin in 1:1 alcohol-ether, in the proportion of about 1 gm. of lipine to 10 c.cm. of celloidin sol. The lipine was suspended (or partly dissolved, in the case of lecithin) by stirring with a glass rod, and the cellodin was then caused to set into a gel by exposure to the vapour of

chloroform. A small piece of the gel was cut out and subjected to the acid-haematein test. The same technique was used as though the celloidin had been a piece of tissue, except that a sliding microtome was used and thicker sections were cut. Microscopical examination revealed that in each case the lipine was in the form of small globules.

The results of these tests are illustrated by Text-fig. 1. The exposure and development of the negative and positive were carried out in exactly the same

Lecithin   Sphingomyelin

Cephalin   Galactolipine
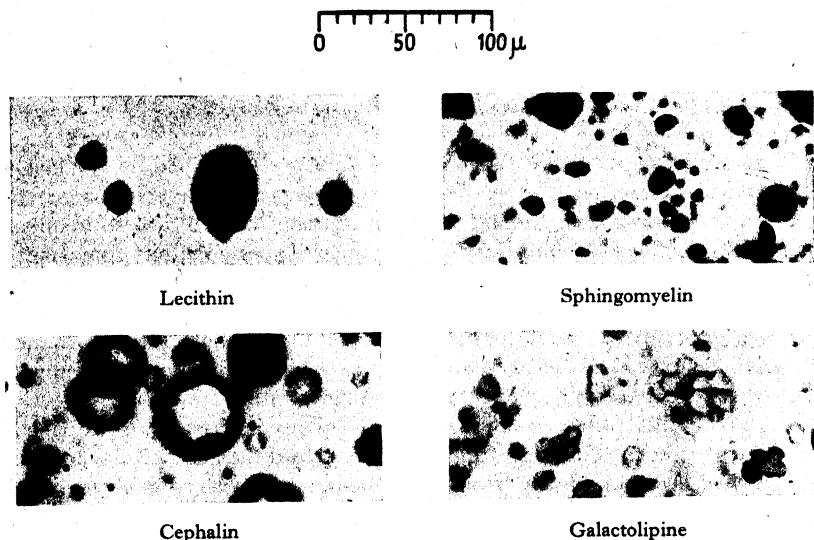
TEXT-FIG. 1

way in each case, so as to make the photographs comparable. The egg-lecithin globules were blue throughout, though sometimes unevenly dark at different distances from their surfaces. Cephalin (from the brains of sheep) gave a different appearance. The centre of each globule was yellow, while the whole of the outer part, or often a layer a little below the surface, was stained blue-black (though some of the globules were yellow all through). A number of the globules were cracked across; when this was so, the cracked surface had an affinity for the dye. Sphingomyelin (crude, from the brains of sheep) gave a different appearance again. The small globules were stained blue or blue-black, while larger ones were yellow in their centres and only had a blue-black rim. The globules of purified galactolipine, which were more uniform in size than those of the other lipines, gave scarcely any trace of a positive reaction. They appeared yellow, sometimes with an extremely faint tinge of blue. When viewed in optical section, their periphery appeared as a very thin dark line, but this coloration could not be recognized when the upper or lower surface of a globule was focused.

To find the cause of the yellow colour seen in the globules, all four lipines

were subjected to a test differing from the standard one in the omission of any treatment with haematein: the sections were treated with 2 per cent. acetic acid instead of the dye solution. In each case the globules now appeared yellow. The darkening of the globules to a yellow colour seems to have occurred mainly during the treatment of the celloidin blocks with hot potassium dichromate.

The results recorded in this paper show that the acid-haematein test, controlled by the pyridine-extraction test, is a method for the histochemical recognition of phospholipine.

Note. The borax-ferricyanide solution used in the acid-haematein test need not be kept in the refrigerator, as was stated in the earlier paper. It suffices to keep the bottle in the dark when not in use.

I thank Prof. A. C. Hardy, F.R.S., for the provision of all the facilities required for the work described in this paper, and Mr. A. J. Cain for the benefit of many helpful discussions.

SUMMARY

1. Carefully purified galactolipine has scarcely any tendency to react positively to the acid-haematein test.

2. It follows that the acid-haematein test (controlled by the pyridine-extraction test) is a method for the histochemical recognition not of lipine in general, but of phospholipine.

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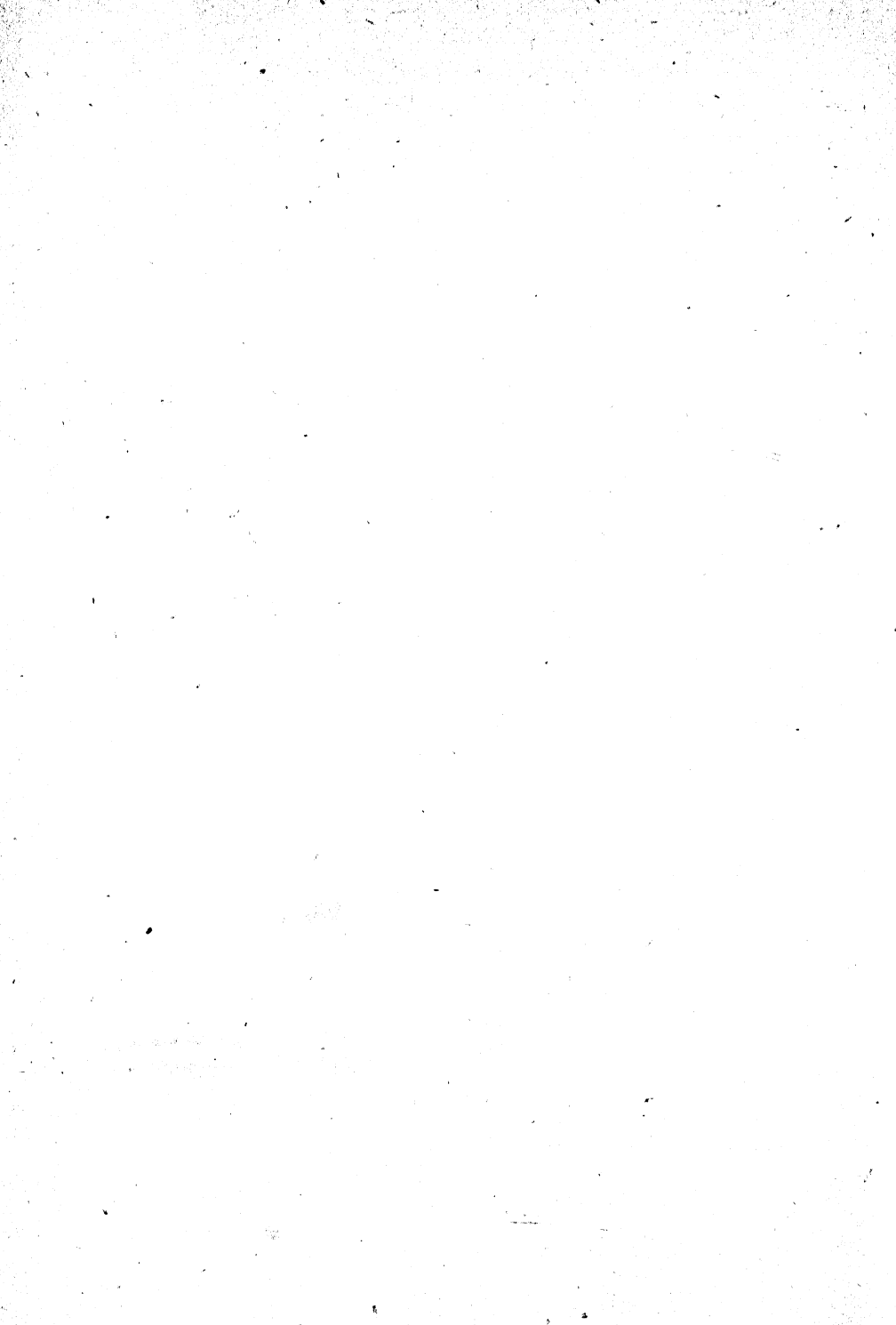
An Examination of Baker's Acid Haematein Test for Phospholipines

BY

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CONTENTS

INTRODUCTION

THE only histochemical test for lipines that was regarded as specific by Lison (1935, 1936) is that of Smith-Dietrich. Baker (1946) has criticized it on the grounds that it is inadequately specified, not very sensitive, and gives a negative reaction with pure lecithin. He proposes his acid haematein test, based on the Smith-Dietrich test but with a pyridine extraction control, as being free from these defects. He has since shown (1947) that the test is for phospholipines only.

It is important to try to assess the value of this test. So many 'histochemical' tests have been shown to be unspecific, and the results obtained by them thereby invalidated, that caution is necessary in admitting the specificity of a new one. It would be better to have no tests rather than unreliable ones.

This paper presents the results of an examination of Baker's test, and gives reasons for believing that, provided only a definitely positive result is considered, it is specific, though there are certain possible sources of invalidity, as Baker has pointed out.

MATERIALS AND METHODS

Transverse sections of the rhynchobdellid leech *Glossiphonia complanata* (L.) were found to give a good variety of tissues, with plenty of lipine in the

fat-cells. Liver, kidney, testis (for interstitial cells) and small intestine of the mouse, and adrenals of the rat were also used. In addition, tests were made on paper impregnated with various pure substances and mixtures. Some of these were repeats of Baker's (1946) tests; all were carried out in accordance with the instructions given by him, except that in some cases the paper was soaked in the melted substance or mixture, and was therefore very thickly coated.

The method employed was to determine what results were given by the acid haematein and pyridine extraction tests when performed in exact accordance with Baker's instructions, and then study the effect of varying the chemical and physical components of the tests. For controls, sections and papers were coloured with sudan black.

Diphenyl-carbazide was used for detecting chromium.

RESULTS AND DISCUSSION

Standard Results obtained with the Acid Haematein and Pyridine Extraction Tests

For convenience, these tests will be referred to as the AH and PE tests respectively. A blue, blue-black, or black coloration given by AH but not by PE indicates phospholipine.

The first point that is noticed on examining an AH section of *Glossiphonia* is that the nuclei, except for the plasmosomes, are nearly colourless. The mitochondria of the nephridial cells, the Golgi apparatus of the stomach epithelial cells, and usually the whole of the cytoplasm of the intestinal cells are blue, as are the cytoplasm of the fat-cells, and granules in the muscle-cells and in certain corpuscles in the coelomic spaces. The epidermis (except for the mucous cells) and the dermis are blue-black or black. Some of the dorsoventral and oblique muscle-fibre cortices may be blue also, but those of the longitudinal fibres vary from colourless through yellow to brown. The interfibrillar spaces in the longitudinal muscles are not usually coloured. The nerve-cords are pale blue, with dark blue in the nerve-cells. The cytoplasm of the salivary gland cells is very dark blue; the pigment cells remain pale brown, as in unstained sections. The cuticle is pale yellow.

Contrasts are seen immediately on examining a PE section. The nuclei are now full of small granules coloured a dark blue-grey. All the blue colorations mentioned above have disappeared completely or have been replaced by a faint blue-grey, except that the epidermis and dermis remain blue-black or black as before. Many of the interfibrillar spaces, particularly near the dermis, are now black or very dark brown. The cortices of the dorsoventral fibres are yellow or dark brown, those of the longitudinal ones, seen in cross-section, may be yellow, brown, black, brown with blue shadings, or deep blue. In one section they may all be yellow; in another, in each bundle there may be some yellow and some dark blue or dark brown. It is possible that the colour may vary with the degree of contraction. The pigment cells are now

full of dark blue granules smaller than those seen in AH and unstained formal-calcium preparations. The nerve-cords still contain some blue. The general tone of the cytoplasm is pale yellow, usually slightly darker than in AH preparations; the cuticle remains pale yellow.

It is evident, then, that in transverse sections of *Glossiphonia* we have examples of all the four classes of substances distinguished by Baker, thus:

(A) AH positive, PE positive. Epidermal cells, plasmosomes.

(B) AH negative, PE positive. Chromatin, pigment granules, interfibrillar matrix.

(C) AH positive, PE negative. PHOSPHOLIPINES. Cytoplasm of fat-cells, salivary gland cells, and intestinal cells, mitochondria of nephridial cells, Golgi apparatus of stomach epithelial cells.

(D) AH negative, PE negative. Fat droplets, cuticle, cytoplasm of nephridial and other cells; and cortex of muscle cells frequently.

The Stages of the Tests

The two tests may be summarized as follows:

AH. (i) Fix in formal-calcium, a non-precipitant fixative specially designed to prevent the loss of lipines (Baker, 1944).

(ii) Soak in potassium dichromate-calcium chloride solution at room temperature and then at 60° C., to make the phospholipines insoluble and mordant them. Wash well in water.

(iii) Cut frozen sections at 10 μ. (Embed in gelatine if necessary.)

(iv) Mordant again in the same fluid at 60° C. for an hour only, as the chromium compound left in the tissues to act as mordant may be washed out of small particles of lipine (Baker, 1946).

(v) Stain in acid haematein for 5 hours at 37° C.

(vi) Differentiate in borax-ferricyanide for 18 hours at 37° C.

(vii) Mount in Farrants's medium.

PE. (i) Fix in weak Bouin to render the phospholipines easily extractable by solvents and increase the power of proteins to give a positive reaction (Baker, 1946).

(ii) Wash in alcohol to remove picric acid.

(iii) Soak in pyridine, first at room temperature then at 60° C., to remove phospholipines (and all other lipoids).

(iv) Wash in water, then go to stage (ii) of the AH test and proceed as for it.

On consideration of these summaries the following questions arise:

(*a*) A calcium-haematein lake is easily produced by adding calcium chloride solution to some acid haematein, alkalinizing, and filtering off and washing the precipitate. A purplish-blue solid is obtained, which turns rusty

red on prolonged exposure to the air. It is insoluble in borax-ferricyanide but dissolves readily in acid haematein. Do the calcium ions in the fixing and chroming solutions assist in mordanting?

(*b*) What evidence is there that a chromium compound is left in the tissues?

(*c*) What is the degree of completeness of extraction by the PE method?

(*d*) Why do some substances stain after PE but not after AH? Obviously something more than a mere extraction has been performed.

(*e*) Does the specificity of the test depend on the period of chroming or on a reaction peculiar to phospholipines?

To which we may add after considering the results described above:

(*f*) How are both blue and brown colorations produced in the finished preparations?

Investigation of the Tests

(*a*) *Role of the Calcium Ions in Fixation and Mordanting.* Variations of the AH test as shown below were tried out to determine the effect of the omission of dichromate and calcium at different stages. All were done on *Glossiphonia* sections.

Variation	Result
1. Fix in 4 per cent. formaldehyde, and omit chroming and differentiation.	Whole section dark reddish-brown. No blue.
2. Same as (1) but with differentiation.	General pale yellow-brown. No blue.
3. Fix in formal-calcium, omit dichromate from the chroming solution, omit differentiation.	General brown. Phospholipine in fat-cells slightly darker brown. No definite blue.
4. As (3) but with differentiation.	Pale brownish-yellow throughout. No blue.
5. Standard AH but with calcium chloride omitted from the chroming solution.	As standard AH results above, but blue reduced greatly. Positive results only in bases of intestinal cells and cytoplasm of a few fat-cells.

The last variation was tried on other tissues also. With rat adrenal, considerably less blue was seen than in standard preparations. With mouse small intestine there appeared to be no reduction in blue-staining, and with mouse liver it was slight.

The conclusions from these results are that:

(i) dichromate is necessary for blue and almost all brown staining in the finished preparation,

(ii) it is necessary for the production of fast browns, and

(iii) calcium ions are necessary with dichromate for the maximum blue staining in some tissues.

Also, the behaviour of various lipoids in thick deposits on paper was investigated in the same way. The following results are representative:

Mixture	Procedure	Colour at start	Colour after staining but before differentiation	Colour after differentiation
Pinene saturated with cholesterol at 60° C.	Standard AH.	White.		General brown. Edges of some crystals medium to dark blue. Scattered dark blue granules.
	AH but omit calcium chloride from chroming solution.	White.	Very pale brown. A few small blue granules.	Pale greenish-brown, no blue.
	AH but omit dichromate.	White.	White.	White.
	AH but omit calcium chloride throughout.	White.	Very pale brown. A few small blue granules.	General pale greenish-brown. A few dark brown granules. A faint lilac tinge on some crystals.
	AH but omit dichromate, and calcium chloride from fixative.	White.	White.	White.

Exactly comparable results were obtained with an equimolecular mixture of cholesterol and oleic acid (BDH redistilled) and with oleic acid freed from all traces of phosphorus. In all cases, after the omission of dichromate no coloration (or only the faintest trace) was seen at any stage, but omission of calcium chloride had no effect. With these mixtures there is no loss of lipoid if calcium is omitted, as there is with phospholipine; so we may conclude that the calcium ions play no part in mordanting here, and it is very improbable that they do with phospholipines.

Examination of formaldehyde-fixed *Glossiphonia* sections with sudan black shows that a considerable amount of lipoid, including some phospholipine, is preserved without treatment with dichromate. Fixation is, of course, rather poor. It is not possible to say from examination of sections that less phospholipine is preserved after 4 per cent. formaldehyde than after formal-calcium, but it is seen from variation (5) on *Glossiphonia* and rat adrenals above that omission of calcium chloride from the chroming fluid does allow phospholipine to escape from some tissue, though not completely. It appears that the short fixation (6 hours) in formal-calcium is enough to fix proteins but not enough to affect the solubility of phospholipines if they are exposed to fluids at 60° C. Baker (1944) found in his studies of lipine solubilities that formaldehyde does make lipines less soluble in various solvents after 24 hours' fixation. One suspects that some lipine is lost even at room temperature if plain formaldehyde is used. If two pieces of lecithin paper prepared by Baker's method (1946) are fixed for 6 and 29 hours respectively in 4 per cent.

formaldehyde, left overnight in water at room temperature, and coloured with sudan black, it is seen that much more lecithin remains on the paper after the longer fixation. Little or none remains on paper fixed for 6 hours and soaked in plain dichromate solution at 60° for 24 or for 8 hours.

We may conclude that:

(i) under the conditions of the AH test neither formaldehyde nor calcium chloride, separately or in combination, will fix phospholipines, and

(ii) the calcium ions merely restrain the phospholipines from passing into solution. (It was for this purpose they were added to the fixing and chroming solutions by Baker.)

(b) *Evidence for a Chromium Compound in the Tissues.* It is seen from variations (i) and (iii) in section (a) above that a dark brown coloration can be produced in the tissues by acid haematein without the aid of dichromate, but it is reduced to a pale yellow-brown by differentiation; no blue is seen. Potassium dichromate is essential for the formation of the fast blue and brown colours; but a chromium lake is not producible by the method described above (part ii) for the calcium lake. Does the dichromate act as a mordant, or are the fast colours produced by the dye and oxidation products in the tissues?

Sections of *Glossiphonia* were prepared (a) as for AH and (b) with formal-calcium fixation but no chroming. Diphenyl-carbazide in 90 per cent. alcohol was used for the detection of chromium as anion. This substance gives colours varying from red to violet with several metallic cations and chromates, arsenates, molybdates, phosphates, and similar anions. (For an account and references, see *BDH Reagents for Delicate Analysis including Spot Tests*, 7th ed. 1939, BDH, London.) The unchromed sections were used as controls. Each section was washed in distilled water, laid on a slide, covered with one drop of a saturated solution of diphenyl-carbazide in 90 per cent. alcohol, and examined at once. (The reagent, if left exposed to the air, gradually goes pink by itself.)

Neither the controls nor the chromed sections gave any colour with the reagent applied alone. On acidifying with acetic acid, the controls still gave no colour, but a fine red was seen at once in the chromed sections. The coloured substance, unfortunately, is alcohol-soluble, and diffuses out rapidly, but on examining the section immediately after applying the reagent it was seen that the colour was most intense just where the deepest staining was seen in AH sections, that is, in the epidermis, intestine, fat-cells, and nerve-cord.

It appears then that a chromium compound, unstable in very acid solution, is to be found in the tissues, most plentifully where the staining is densest. The acid of the dye may have a slight differentiating effect on the mordant, perhaps.

As a confirmation, a paper test on lecithin and caseinogen, both of which give deep blue with AH, was carried out, unimpregnated paper being used as the control. All the papers were put through the AH test without section-

ing, the acid haematein being replaced by 2 per cent. acetic acid. After 24 hours' washing, both the lecithin and caseinogen papers gave strongly positive results and the controls were colourless. Washing for 2 more days reduced the colour in both cases but did not abolish it. (These tests might perhaps be criticized on the grounds that as it took 24 hours' washing before the controls were free from chromium compounds some chromium may have been trapped inside the papers under the layers of lecithin or caseinogen; the reduced reaction on further washing is not necessarily evidence that the chrome-lecithin or chrome-caseinogen compound is removed by washing.)

Pieces of bath-sponge stain deep brown after both AH and PE. After AH with no mordanting they stain deep brown but lose almost all their colour during differentiation. Mordanted pieces give a strongly positive result with diphenyl-carbazide and glacial acetic acid, but slowly. Also, if lecithin paper, unchromed, is stained in acid haematein, most of the lecithin is lost and there appears to be no staining. If acid haematein made up with 1 per cent. calcium chloride instead of distilled water is used, there is still no staining and little if any lecithin is lost.

Apparently a chromium compound is part of both blue- and brown-coloured substances. (The blue colour with dichromate and lipoids has, of course, been considered a chrome lake for very many years.) The above experiments do not rule out the possibility of the chromium combining with oxidation products of the lipoids rather than with the lipoids directly. Smith and Mair (1909) have shown that oleic acid will form an intermediate chrome compound in the course of oxidation to a dioxystearic acid.

(*c*) *Extraction by Pyridine.* The test of this is very simple. A *Glossiphonia* section was produced as for the PE test but was coloured with sudan black instead of acid haematein, and compared with an AH section similarly treated. The result shows that the extraction is very good. An exceedingly faint coloration is visible in places where phospholipine was very plentiful (notably in the fat-cells), and the rest is uncoloured. The contrast with the sudan-coloured AH section is most striking.

(*d*) *Substances staining after PE but not after AH.* From what has been said so far it is understandable that substances should be AH +ve and PE −ve, Ah +ve and PE +ve, or AH −ve and PE −ve, but it is a little difficult to see how the combination AH −ve and PE +ve can occur. The differences between the two tests are:

(i) the use of acetic and picric acids in the PE fixative;

(ii) the omission of calcium chloride from the PE fixative; and

(iii) the use of the strong base pyridine in the PE test for extracting lipoids.

This subject was not followed up in detail, but some points should be mentioned.

In *Glossiphonia* sections prepared as for PE but with the actual extraction omitted, the pigment-cell granules are much smaller and darker than in AH

sections, and nuclei, having been treated with a powerful protein precipitant, show dark granules of chromatin. It seems likely that in these cases the difference between AH and PE results is due to non-precipitant and precipitant fixation respectively. Baker has noted (1944) that chromatin in thick layers is coloured by AH. The colour of the pigment cells is probably entirely intrinsic and not due to staining.

The case of the interfibrillar matrix is more puzzling, but it should be noted that in exceptional instances it is coloured after AH. It is invariably coloured after PE but to a depth varying greatly in different individuals. In PE-fixed sections without extraction and chroming the pigment-cell granules are very dark (which confirms that their coloration is by concentration of their own pigment) but the rest of the section is a general yellow-brown, as in a PE section with only the extraction omitted. This seems to mean that the pyridine has some effect on the interfibrillar matrix, but the results vary in different individuals.

(e) *Specificity and the Period of Chroming.* Smith and Mair (1909) have investigated the action of dichromate solutions on lipoids, and their results are of great interest here. They found that many unsaturated lipoids would give a blue colour with haematoxylin after chroming for various periods, and even saturated ones could do so if mixed with cholesterol, which by itself was negative. The necessary period of chroming for unsaturated lipoids was shortened by adding cholesterol to them, in a few cases to 24 hours. With lecithin they obtained a negative result, almost certainly because, as Baker has suggested, there being no calcium ions present it went into solution. Kaufmann and Lehmann (1926), on examining the Smith-Dietrich test, considered it specific if a black colour only were taken as positive; blues, greys, and browns were given by various mixtures not containing lipines. Baker reduced the concentration of the stain considerably so that these weaker, non-specific results might be abolished. Two of Kaufmann and Lehmann's mixtures containing cholesterol or cholesteryl oleate but no lipines gave positive results, and Lison (1936) concludes that unless cholesterol or cholesterides can be excluded there remains a slight doubt. Smith and Mair found that a cholesterol-oleic acid mixture gave a clear blue after 24 hours' chroming at 65° C. with saturated potassium dichromate solution.

The following lipoids were therefore investigated, thick coatings on paper being used:

Oleic acid (phosphorus-free).
Cholesterol and oleic acid (BDH redistilled) in equimolecular proportions.
Cholesterol dissolved to saturation in pinene at 60° C.
Cholesterol dissolved to saturation in tributyrin at 60° C.

Details of the cholesterol-pinene results are given in part iii above. Cholesterol by itself is uncoloured by AH, but is solid at the temperatures of the test. All the above gave a few granules of pale or medium, and sometimes dark blue. Occasionally edges of thick masses of crystals were tinged bluish or

lilac. In the case of the oleic acid, staining produced a definite purple or purplish-blue which was almost completely removed during differentiation.

As was mentioned above, Kaufmann and Lehmann found that very many mixtures gave faint colours after the Smith-Dietrich test. Unfortunately, consideration of their results with nile blue (Cain, 1947) shows that some of their substances, including triolein or cholesterol in one case, were not pure. (It is worth noting here that their specimens of phrenosin and kerasin gave heavily positive results in mixtures containing no other lipine, and faint ones by themselves, with the Smith-Dietrich test. Baker has shown (1947) that a mixture of phrenosin and kerasin carefully freed from all traces of sphingo-myelin (the most difficult of the phospholipines to remove) is negative to AH.) Consequently, their detailed results must be accepted with reserve, though supported in general by Smith and Mair's results and by those given above. At the same time, it must be remembered that they were the first to use extensive tests on pure substances and mixtures to assess the validity of histochemical methods for lipoids.

The weak colorations obtained with AH in mixtures on paper were never seen throughout the coating, and this was very thick, probably of the order of 100 or 200 μ, whereas the coloration of lecithin paper prepared according to Baker's instructions is present wherever there is lecithin on the paper and is quite a dark blue, although the total thickness is only about 10 μ (Baker, 1946). Very occasionally, as in a cholesterol-tributyrin preparation, there is a small cloud of granules of a medium blue but again only locally in a mass of the mixture far larger than could be expected in tissues. After study of the results it is concluded that a definitely positive result (dark blue or blue-black) with AH but not with PE does indicate phospholipines, but that with weak results (pale blues and greys) there is no certainty, unless, of course, a chemical ana-lysis of extracts of the tissues shows that phospholipines are the only lipoids present. The intense coloration of mitochondria in many cells gives a good example of a positive result in very small bodies.

Prolonged chroming has been shown by Smith and Mair to produce blue colours with haematoxylin in many lipoids. (However, on repetition of one of their results, it was noted that chroming of the cholesterol-oleic acid mixture for several days gave with AH an increase in brown coloration but not in blue.) The specificity is therefore dependent on the period of chroming, which must never be increased. With AH, decreasing the period caused an increased response in the mitochrondria of mouse liver but a decreased one in those of mouse small intestine and kidney, and appeared to have no effect on the pale blues of the cholesterol-oleic acid mixture. It is therefore not recom-mended, as it may weaken the results obtained with some tissues and make them uncertain. A preliminary extraction with acetone to remove interfering lipoids was tried and gave fairly good but definitely fainter results and con-siderably increased the length of the test.

(*f*) *The Blue and Brown Colorations.* It was shown above (*b*) that a chromium compound is formed in the tissues in both blue- and brown-staining. If an

undifferentiated *Glossiphonia* section is examined, it is seen that what is blue in the differentiated section is blue there also and in much the same depth of colour. Differentiation, even for 88 hours, makes little difference to a *Glossiphonia* section as far as the blue stain is concerned. This is not true of the brown. In the undifferentiated section all that is not blue is dark brown except ground cytoplasm, which is nearly colourless or pale yellow. The effect of differentiation is to reduce the dark brown to pale yellow-brown or yellow. After 88 hours' differentiation, plasmosomes in the nuclei of intestinal cells have changed from black to brown. If, however, the progress of the AH test is observed with pure oleic acid (see (*e*) above) it is seen that a blue or purple colour is produced which is removed by differentiation. Consequently, it is important to keep to the prescribed period of differentiation. Shortening is not permissible; lengthening seems to have little effect on phospholipines in general, but might remove the colour from very small objects or weaken it to the point of uncertainty.

It will be noticed from Baker's results of paper tests that all the very dark blue-staining substances except legumin contain phosphorus. It is quite probable that such substances as fibrinogen, blood-albumen, mucin, nucleo-protein, nucleic acid, and perhaps trypsin might be impure. A faint positive result might almost be expected with blood-serum (rabbit) and blood-plasma (fowl). However, sodium glycerophosphate gave a negative result. But this substance dissolves in both formal-calcium and dichromate-calcium, so it is likely that it was lost from the paper at an early stage and the negative result is not significant. A negative result with a paper test means nothing unless it is shown that the substance was still present at the end of the test or that it would have been lost from tissues. A small quantity of this salt was dissolved in dichromate-calcium and the solution incubated at 60° C. for 18 hours as for chroming for AH. At the end of that time a crystalline precipitate had formed which was removed by filtration, when it was seen to be a very pale yellow, almost white. It was only slightly soluble in water, and a small portion tested with diphenyl-carbazide gave a faint red at once which was greatly increased on acidifying. (The first faint colour was almost certainly due to the retention of a little mother-liquor.) The rest was stained and differentiated as for the AH test. The resultant colour was black. This substance resembles the blue-stained substances in AH sections quite closely in its formation. One might suggest that rapid and intense blue staining is usually due to some combination between chromium and substituted phosphoric acids.

But this will not explain the case of legumin, and would lead one to suppose that at least some nucleoproteins would stain heavily, which is not so. Baker notes that all three dark-blue staining proteins (caseinogen, mucin, and legumin) are very acidic. Stearic, palmitic, and ricinoleic acids all gave a negative or exceedingly faint positive result, so the mere presence of an acidic group is not sufficient. Smith and Mair's work with oleic acid appears to show that chromium enters into combination at the double bond; prolonged

chroming abolishes the capacity to stain, a dioxy-compound being formed which contains no chromium.

The only safe conclusion appears to be that with the AH test, blue and brown colours are produced, both fast to 18 hours' differentiation with borax-ferricyanide at 37° C. The dichromate acts as a mordant in the blue staining and some at least of the brown, though it is not proved that in some cases it may not cause oxidation first. Under the conditions of the AH test mordanting takes place most readily with certain acidic compounds, especially some containing phosphorus. (This suggests that interference might come from bound phosphorylated sugars and other compounds in tissues if they are removed only by the action of pyridine.)

The production of different colours according to the substrate by the same dye *and mordant* under the same conditions has not, to my knowledge, been emphasized before, and seems worth investigation.

Summary

1. Baker's acid haematein test for phospholipines is specific provided that only a definite positively result is considered. Very pale blues and greys may be caused by other lipoids, which if present in very large masses may possibly show medium to dark blue granules but will not be coloured all through.

2. The mechanism of the test appears to be as follows:

(*a*) Phospholipine is not fixed by formal-calcium but is restrained from passing into solution by the calcium ions, which play no other part.

(*b*) Phospholipine combines readily with chromium from the mordanting fluid, and is then rendered insoluble and mordanted. Other substances, acidic and usually containing phosphorus, are mordanted as well.

(*c*) On staining, blue and brown colorations are formed; in both cases the dye attaches itself to the chromium in the various substrates.

(*d*) On differentiation, some browns and most blues, particularly those with phosphoric substrates, remain nearly fast, but most browns and the weak blues of certain lipoids (not phospholipines) are greatly reduced or removed entirely. The period of differentiation must not be shortened.

(*e*) Blue-staining lipoids (phospholipines) are distinguished from other blue-staining substances by an extraction with the lipoid solvent pyridine, after special fixation. The other substances, and any bound lipoid not removable with pyridine, remain.

3. Since the specificity of the test depends on the *relatively* greater affinity of phospholipines among lipoids for the mordant, the period of chroming must not be lengthened.

4. One reason why some substances are coloured after pyridine extraction but not after acid haematein is that in the former case they are precipitated and so concentrated; in the latter they are not. This is not a general explanation for the whole class of such substances.

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A Comparative Histological Study of Haemosiderin in the Uteri of Mice of Cancerous and Non-cancerous Strains

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With 3 Text-figures and 3 Plates

Contents

INTRODUCTION

WITHIN the uterine wall in mice large masses of a brown or yellowish-brown granular substance are often observed. This has been identified as haemosiderin—a pigment derived from the breakdown of haemoglobin. This substance contains iron and therefore gives the well-known 'Prussian blue' reaction on treating with potassium ferrocyanide and hydrochloric acid.

In the course of the comparative histological examination of uteri of mice of cancerous and non-cancerous strains, it soon became apparent that, although masses of haemosiderin occurred in the uterus of almost every mouse of the cancerous strain, such large masses were not observed in the uteri from the non-cancerous strain. Further study, however, showed that the pigment is formed in both cancerous and non-cancerous strains from the blood which is extravasated into the tissues of the uterus at parturition, but in the cancerous strain it is formed somewhat more slowly and persists much longer than in the non-cancerous strains examined. The evidence for these differences, their nature, and their possible significance form the subject-matter of the present paper.

MATERIAL AND TECHNIQUE

The strains of mice employed in this investigation included cancerous and non-cancerous strains. The cancerous strain—the R III albino strain of Dobrovolskaia-Zavadskaia—showed a very high incidence of mammary carcinoma, in females over 7 months old, at the time of investigation (1940). This strain was bred in this department as two sub-strains, both cancerous, the CB and CBB strains. Another albino strain, non-cancerous, the Wistar strain, has been inbred for over 4 years in this department without ever developing cancer. A recent non-cancerous strain, of piebald mice, the P strain, has been inbred for almost 5 years, again without showing any cancer development. The PC strain, also piebald, and the XL strain, self-fawn and self-brown, have both been entirely cancer free during the 3 years that they have been inbred in this department. All the uteri used for study have been fixed in either Bouin's fluid or formol-saline solution (9 per cent.). In order to prevent muscular contraction of the uterine walls the whole female reproductive apparatus was dissected out and pinned, in a stretched position, on a layer of paraffin wax in a shallow dish into which the fixing fluid was then poured. After fixation, dehydration, and clearing, the right and left horns of the uteri were embedded separately.

Microscopic sections were cut 5μ in thickness, and stained, for ordinary histological study, with Ehrlich's haematoxylin and eosin. Alternate slides of the serial sections were treated with potassium ferrocyanide and hydrochloric acid, after prolonged contact with nitric acid alcohol, and then counterstained with eosin. The details of this method are given by Bolles Lee (1937) and are as follows: The sections, after removal of the wax, are placed in nitric acid alcohol (3 per cent. in 95 per cent. alcohol) for 36 hours at 35° C. They are then washed in 90 per cent. alcohol and in distilled water. After washing, they are placed, for not more than 5 minutes, in a freshly made solution composed of equal parts of 1·5 per cent. potassium ferrocyanide in water and 0·5 per cent. hydrochloric acid, also in water. The sections are again washed carefully in distilled water, stained for 3 minutes in eosin (1 per cent. in 30 per cent. alcohol), differentiated in 90 per cent. alcohol, cleared in cedarwood oil, and mounted in benzene balsam. By this method haemosiderin is stained a bright Prussian blue.

DATA

Description of Haemosiderin and its Location in the Uterus

In transverse sections of some uteri a large mass of brown or yellowish-brown material is seen situated dorsally in the thickness of the wall just below the mesentery (Text-fig. 1). Serial sections reveal that these dorsal masses are present only at intervals.

Occasionally there are also in the uterine wall smaller scattered clumps of the material situated at the inner and outer borders of the circular muscle layer. Such scattered material has been observed in only a few of the uteri examined and has not been included in the present study. Where it occurs

at all it is present all along the uterus, not at intervals as are the dorsal masses. It has never been seen in uteri which do not contain dorsal masses, and in only a very small proportion of those that do.

Under the high power of the microscope the material is seen to be granular in consistency, the granules being arranged in small ovoid groups or clumps (Pl. I, figs. 1 and 2) which tend to aggregate to form larger masses (Pl. I, fig. 2). These small ovoid groups appear to be cell-bodies crowded with the granules, since each contains a nucleus (Pl. I, fig. 1). The granular material in form and natural colour agrees with the published descriptions of haemosiderin,
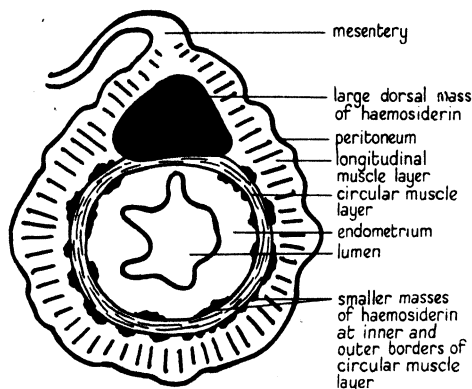
TEXT-FIG. 1. Diagram of a transverse section of mouse uterus a few months after parturition, to show distribution of haemosiderin.

and on staining with potassium ferrocyanide and hydrochloric acid it gives the typical Prussian blue reaction.

Origin of the Pigment

As haemosiderin is known to be the pigment commonly formed when there has been extravasation of blood into the tissues, it was thought that possibly the haemosiderin present in the uterine wall is derived from the blood extravasated at parturition. The large masses of haemosiderin are found only in the dorsal wall of the uterus, close to the mesentery—a location identical with that of the placentae (cf. Pl. II, figs. 3 and 4); the masses occur at intervals along the uteri, as do the placentae; these masses were never found in virgin uteri, and when the breeding records were examined for the animals under investigation it was found that in every case where these masses of haemosiderin occurred the animal had borne young. Finally, by examining a

complete series of sections the number of masses of haemosiderin could be counted, and on referring to the breeding records it was found that, where only one litter had been produced, the number of masses of haemosiderin corresponded in every case with the number of young in the litter. It was then found that these dorsal masses of pigment could be seen with the naked eye as dark spots situated close to the mesentery when the whole uterus was cleared in cedarwood oil (Pl. II, fig. 5). (Uteri to be examined in this way should be fixed in formol-saline or formaldehyde as these clear much more perfectly than Bouin material.) The number of these dark spots in every case corresponded exactly to the number of young born, when the mouse had had only one litter. Uteri taken immediately or very shortly after parturition also show similar dark spots when cleared. Pl. II, fig. 7, shows a uterus, from the non-cancerous strain, taken 2 days after parturition, and Pl. II, fig. 8, a cancerous strain uterus also 2 days after parturition. When sectioned, however, these spots are found to contain very little haemosiderin, if any. Each is composed of a mass of extravasated blood and torn tissue which marks the former site of the placenta. These spots are very large, but by 25 days they are much smaller, as can be seen in Pl. II, fig. 5, which shows a cancerous strain uterus taken 25 days after parturition. This decrease in size is caused by a healing of the torn tissues. Sections then show that it is chiefly a quantity of haemosiderin which marks the site of the placentae. Later, uteri from mice which had been pregnant more than once were examined, and it was often found that the sites of the placentae of successive pregnancies could be distinguished from one another by the different size of the dark spots, since the placentae of one pregnancy did not overlap with those of another. The dorsal masses, when present, from two successive litters could always be distinguished when only a few days had elapsed since the last pregnancy, as seen in Pl. II, fig. 6. This is a photograph of the cleared uterus of a mouse of the cancerous strain, 3 days after parturition, which has had two litters with seven young in each. In the photograph, seven small dark spots and seven larger ones are to be seen situated close to the mesentery. Here the seven large spots

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PLATE II

Fig. 3. T.S. of uterus of mouse (of cancerous strain) containing a large amount of haemosiderin. Fe (K ferrocyanide + HCL) and eosin. Low power. × 74.

Fig. 4. T.S. of uterus of mouse almost immediately after parturition, showing extravasated blood at site of placenta. Ehrlich's haematoxylin and eosin. Low power. × 21.

Fig. 5. Whole uterus of mouse (of cancerous strain) which has had only one litter. Cleared in cedarwood oil. × 3½.

Fig. 6. Whole uterus of mouse (of cancerous strain), 3 days after parturition, which has had two litters. Cleared in cedarwood oil. × 3½.

Fig. 7. Whole uterus of mouse of a non-cancerous strain 2 days after its first parturition. Cleared in cedarwood oil. × 3½.

Fig. 8. Whole uterus of mouse of the cancerous strain 2 days after its first parturition. Cleared in cedarwood oil. × 3½.

Fig. 9. Whole uterus of mouse of a non-cancerous strain 10 months 7 days after its first parturition. Cleared in cedarwood oil. × 3½.

Fig. 10. Whole uterus of mouse of the cancerous strain over 10 months after its first parturition. Cleared in cedarwood oil. × 3½.

blood vessel

erythrocytes

phagocyte containing fine
granules of haemosiderin

large phagocyte containing
fine granules and clumps
of haemosiderin

nucleus

nucleus

mass of haemosiderin-
containing phagocytes

isolated phagocytes
containing haemosiderin
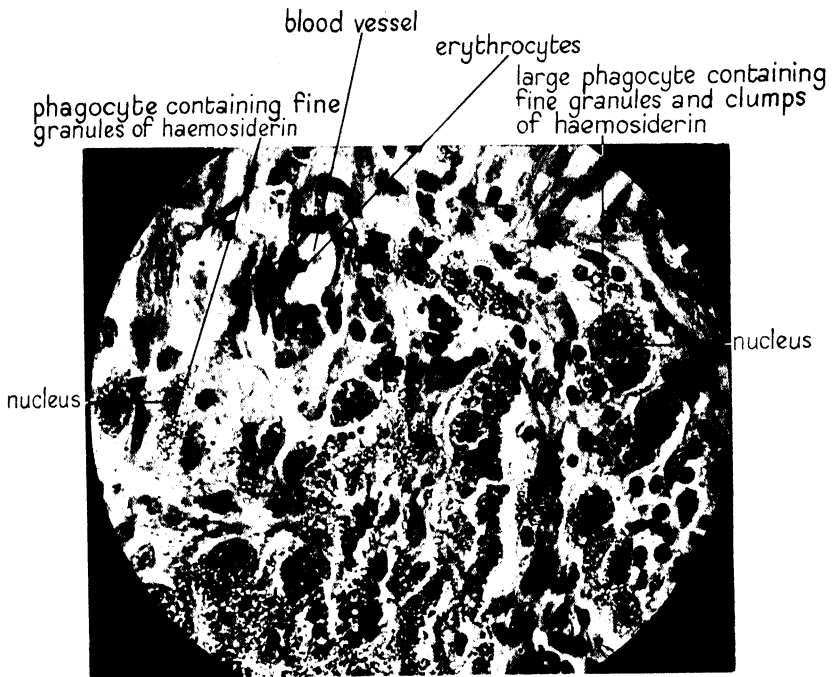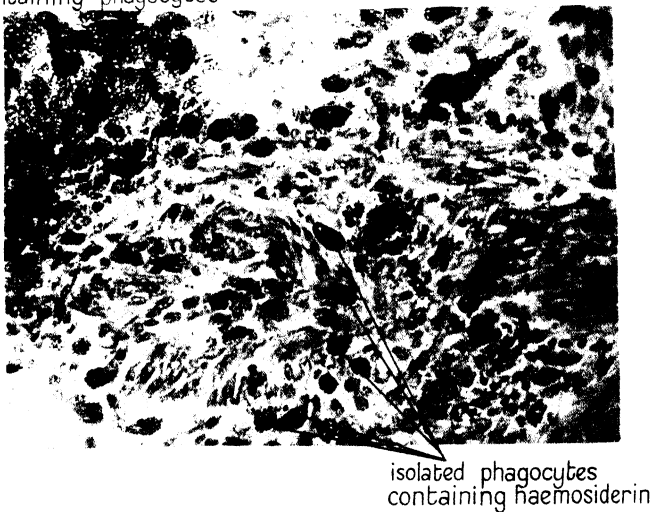
mesenteric wall
of uterus

large dorsal mass
of haemosiderin –
containing phagocytes

mesenteric
wall of uterus

former site
of placenta
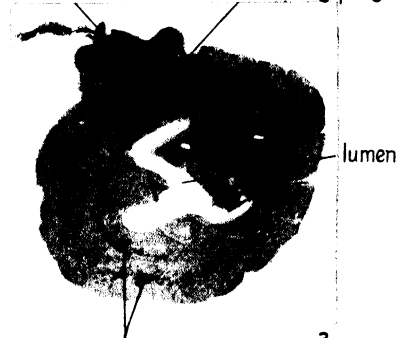
lumen

smaller masses at inner
and outer borders
of circular muscle

3

blood shed at
parturition

lumen

4

ovary
uterus
small dark spots
in mesenteric
wall of uterus
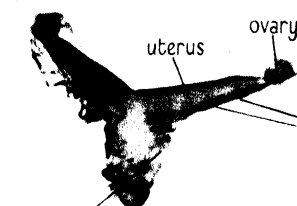
mesentery with
blood vessels

5
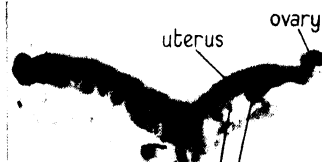
ovary
uterus

large spot and smaller spot in
mesenteric wall of uterus

6
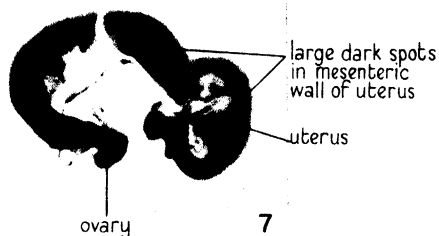
large dark spots
in mesenteric
wall of uterus

uterus

ovary

7

ovary

uterus

large dark spots in
mesenteric wall of uterus

8

ovary

uterus

9

uterus

ovary

dark spots in
mesenteric wall
of uterus

10

(which represent the placentae of the last pregnancy) are composed chiefly of blood and torn tissue, and the seven small spots (which represent the placentae of the previous pregnancy) consist of haemosiderin.

It seems abundantly clear, therefore, that the dorsal masses of haemosiderin under consideration are formed, after parturition, in the regions of former attachment of the placentae—probably from the blood extravasated into the tissues when the placentae break away at parturition.
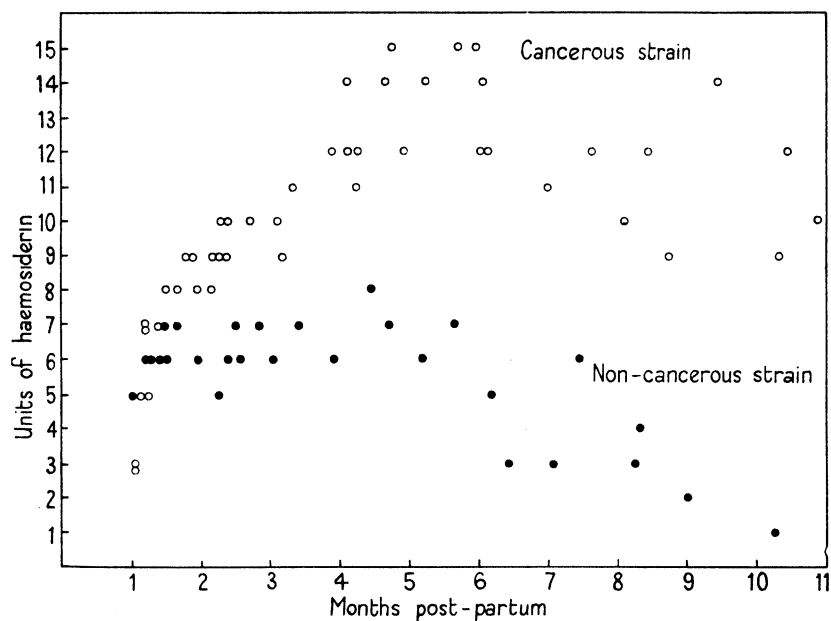
TEXT-FIG. 2. Relative amounts of haemosiderin at various periods after parturition in the uteri of cancerous and non-cancerous mice.

Incidence and Relative Amounts of Haemosiderin in Uteri of Mice from Cancerous and Non-cancerous Strains at Various Times after Parturition

Since there was the ever-recurring suggestion that the cancerous strain shows more haemosiderin or shows it more frequently than the non-cancerous strain, a survey was made of the uteri of mice of different strains taken at various times after parturition, from 1 month to 10 months. The results of this survey are shown in Text-fig. 2. No attempt has been made to indicate the exact amounts of haemosiderin present—the estimates are very rough indeed. One unit represents the smallest amount of haemosiderin found in any one uterus, and therefore Text-fig. 2 gives *relative* amounts only. Where the haemosiderin is compact, the area of the largest section of the mass has been measured: where it is less compact, the area of the various parts have been measured. No attempt has been made to calculate the volume, so that

the figures given understate the difference in amount. In actually examining the material the differences are very striking indeed.

From the data given in Text-fig. 2 it is evident that almost throughout the series there is much more haemosiderin in the uteri from the cancerous strain than in those from the non-cancerous, taken at corresponding times after parturition. The difference is not apparent, however, till about $1\frac{1}{2}$ months after parturition. Up to that time the uteri of the cancerous strain contain about the same amount of pigment as those of the non-cancerous strains —possibly less. After about $1\frac{1}{2}$ months the amount increases very considerably in the cancerous strain, until about 6 months, but not appreciably in the non-cancerous. After 6 months there is a very definite decrease in amount in the non-cancerous strain but very little decrease in the cancerous strain.

These facts are clearly illustrated in Pl. III, figs. 11 to 15. At 1 month 6 days after parturition (Text-fig. 2) the two uteri (of cancerous and non-cancerous strains) contain approximately the same amount of haemosiderin, although in the non-cancerous strain the pigment is more dispersed, but at 3 months after parturition the cancerous strain uterus (Pl. III, fig. 12) shows a very great amount of haemosiderin as compared with that of a non-cancerous strain (Pl. III, fig. 11). The pigment in the non-cancerous strain uterus is spread over as great an area as that in the cancerous strain, but again is very loosely distributed. Pl. III, fig. 13, shows a uterus of a non-cancerous strain 7 months after parturition and Pl. III, fig. 14, shows one from a cancerous strain at 8 months. Again, the haemosiderin is very much greater in amount in the cancerous strain, and much more compact. By 9 months after parturition the non-cancerous strain uterus shows only a very small amount of haemosiderin (Pl. III, fig. 15) and by 10 months only the merest trace remains —not sufficient to show satisfactorily in a photograph, whereas in the cancerous strain the uteri still show a very large amount of pigment. This decrease in the amount of haemosiderin in the non-cancerous strain and its retention in the cancerous strain is borne out by examination of cleared whole uteri taken at 10 months and more after parturition. In the non-cancerous-strain uterus shown in Pl. II, fig. 9, taken 10 months after parturition, there is no trace of the dark dorsal masses (the small trace found in sections is not visible to the naked eye), whereas in the uterus of the cancerous strain taken after a much longer period (exact date unknown, but at least 10 months) the masses are still clearly visible as dark spots close to the mesentery (Pl. II, fig. 10).

It seems to be clearly established, therefore, that at all times after parturition, apart from about the first month and a half, as long as haemosiderin is present at all, it is present in greater quantities in the cancerous-strain uteri than in the uteri of the non-cancerous strains. Further, it seems quite clear that it disappears almost completely after about 10 months in the non-cancerous strains examined, whereas in the cancerous strain it is scarcely diminished in amount at all at that time.

These findings raise several interesting questions. Is less haemosiderin formed in the non-cancerous strains? Or is it dispersed more quickly as it is formed, so preventing the accumulation of large quantities? Is it dispersed at all in the cancerous strain or is it merely more tightly packed as time goes on? In an attempt to throw light on these questions an investigation of the method of formation of haemosiderin was undertaken.

Mode of Formation and Fate of the Pigment in Non-cancerous Strains

For an investigation of the method of formation of haemosiderin within the wall of the uterus, only material from non-cancerous strains was used in the first place, and it is an account of the process as observed in this 'normal' material which is given below.

Immediately after parturition the former site of the placenta shows a considerable quantity of extravasated blood and, a little later, numerous phagocytes can be distinguished ingesting the blood. Cells containing ingested red corpuscles are first observed in uteri taken 12–24 hours after parturition. Ingestion of the erythrocytes seems to be followed within 48 hours after parturition by the appearance of haemosiderin, firstly in a diffuse form and then granular. In material taken at about 48 hours after parturition and stained with hydrochloric acid and potassium ferrocyanide, the phagocytes containing ingested erythrocytes show a diffuse blue coloration of their cytoplasm (Text-fig. 3*a*), and in slightly later material they show minute darker blue granules in the cytoplasm (Text-fig. 3*b*). This suggests that after ingestion some of the haemoglobin diffuses out of the erythrocytes and is broken down into diffuse pigment which is then converted into small granules of haemosiderin. In later material again, stained in the same way, blue-staining non-granular bodies are observed within the phagocytes, similar in shape and size to the erythrocytes (Text-fig. 3*c*) and later still, similar bodies, but of a granular consistency, are to be seen in the same situation (Text-fig. 3*d*) —which seems to indicate that the haemoglobin remaining within the erythrocytes is broken down, *in situ*, into haemosiderin which is at first in a diffuse state and later becomes granular. Phagocytosis of the red corpuscles continues until all extravasated blood is removed from the tissues. This takes a considerable time and cells containing unaltered red corpuscles have been observed as late as 21 days after parturition. Meanwhile, deposition of granular haemosiderin increases day by day until the phagocytes are crowded with the pigment (Pl. I, fig. 1). By 21 days a considerable amount of haemosiderin can be seen in the uterine wall and this continues to increase until about a month and a half after parturition. After this time little or no increase has been observed (see Text-fig. 2, non-cancerous). Increase in quantity of haemosiderin is accompanied by a close aggregation of the pigment-forming phagocytes. These cells, at first widely dispersed, gradually come together into small groups which aggregate to form a loosely packed mass. It should be emphasized that in this non-cancerous material the pigment is never very closely packed (see Pl. III, figs. 11 and 13). No extracellular formation of

haemosiderin has been seen. All the pigment has been found within cells except in extravasations of long standing where a small amount of extracellular pigment is present, almost certainly due to disintegration of some of the phagocytes.

It is of interest to compare these observations with those of Muir and Niven (1935). These workers injected fresh mouse blood into mice and
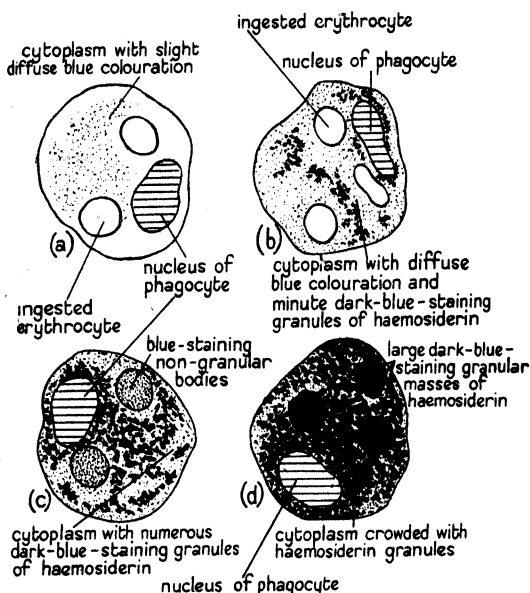
TEXT-FIG. 3. Four stages in the formation of haemosiderin within the normal mouse phagocyte. Drawings made from T.S.s of uteri. Fe (K ferrocyanide + HCl) and eosin. Oil immersion. × 2,700.

studied its fate in the tissues. They observed phagocytosis of red corpuscles within 24 hours after injection and also the appearance of diffuse and granular haemosiderin within the phagocytes from 24 hours onwards. They, too, observed both small granules and large granular masses of haemosiderin and also ingested erythrocytes which gave the Prussian blue reaction. They also

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PLATE III

Fig. 11. T.S. of uterus of mouse of a non-cancerous strain 3 months 28 days after its first parturition. Fe and eosin. Low power. × 40.

Fig. 12. T.S. of uterus of mouse of the cancerous strain 3 months 5 days after its first parturition. Fe and eosin. Low power. × 40.

Fig. 13. T.S. of uterus of mouse of a non-cancerous strain 7 months 12 days after its first parturition. Ehrlich's haematoxylin and eosin. High power. × 275.

Fig. 14. T.S. of uterus of mouse of the cancerous strain 8 months 12 days after its first parturition. Fe and eosin. High power. × 275.

Fig. 15. T.S. of uterus of mouse of a non-cancerous strain 9 months after its first parturition. Fe and eosin. High power. × 275.

state that they observed only intracellular formation of the pigment. Muir and Niven go on to say that at about the seventh day after injection of the blood haematoidin appears in the bodies of the phagocytes and gradually replaces the haemosiderin from which they believe it to be derived. Our own findings, however, show that haemosiderin is the only pigment formed in the mouse uterus from the extravasated blood after parturition: it can still be demonstrated after 10 months in small traces and throughout that period there is no suggestion of haematoidin or of any other pigment anywhere in the uterus. Muir and Niven state that in the period covered by their observations (24 hours to 36 days after injection) they found no haematoidin in phagocytes situated close to blood-vessels or to nerves, haemosiderin being the only pigment found in the cells in these localities, and they add that in the rabbit there was no formation of haematoidin up to 5 days, when their observations ended.

The fate of the haemosiderin in our own material is not known with certainty, but there is a strong suggestion that the phagocytes carry it away from the site of formation. They are never found in the tissues of the uterus surrounding the region of formation, but they are found in the mesentery (Pl. III, fig. 11). It is not certain that they have migrated there from the dorsal mass, but the appearance strongly suggests that they have done so. The dorsal mass seems to 'tail off' into the mesentery and to be continuous with a 'stream' of phagocytes within the mesentery. At the same time the appearance of the dorsal mass changes in a way which suggests bodily removal of some of its constituents. The actual size of the region in which the haemosiderin occurs remains about the same, but the material looks more loosely packed as time goes on until, when only a trace of the pigment remains, it is seen, in section, as tiny clumps of granules scattered over the original area as in Pl. III, fig. 15. Dark blue granules are visible but nothing is clear-cut —they seem to be slightly bordered by a pale blue diffuse stain. On first examining sections in this condition it was thought that the method of preparation had partly dissolved the granules, but this is not the case: every section showing the haemosiderin during its late history shows this blurred appearance. It looks as though there might be a slight diffusion of the pigment immediately around the granules, but the matter is very uncertain. The whole picture of the process, as gleaned from sections, much more strongly suggests bodily removal of the phagocytes with their loads of pigment.

Comparison of the Cancerous and Non-cancerous Strains, with regard to the Mode of Formation and Subsequent History of Haemosiderin in the Uteri

In the present study, after the process of formation of haemosiderin and its subsequent disappearance had been followed in the uteri of non-cancerous strains of mice, attention was turned to the process in the cancerous strain. The basic procedure was found to be the same in both, but certain differences stand out very clearly. In the first place, ingestion of red corpuscles by the phagocytes seems to be slower in the cancerous than in the non-cancerous

strains. In the former, it does not begin at the earliest until after 24 hours after parturition—in some not until after 48 hours; whereas in the non-cancerous strains it is clearly marked between 12 and 24 hours. Further, in the cancerous strains phagocytosis can be observed to be still taking place a month or so after parturition, whereas in the non-cancerous strains no trace of it is found after 21 days. The appearance of haemosiderin is also delayed in the cancerous strain until nearly 3 days, sometimes until 4 days, after parturition, whereas in the non-cancerous strains it is unmistakably present at 48 hours. The amount of haemosiderin gradually increases, but again very much more slowly in the cancerous strain. Comparison between a cancerous-strain uterus and a non-cancerous-strain uterus at 21 days after parturition shows very much less haemosiderin in the cancerous strain. But by 1 month 6 days the two strains show approximately the same amount of pigment, although it is more compact in the cancerous strain than in the non-cancerous.

The later history of the pigment in the two strains has already been dealt with above (see p. 483 and Text-fig. 2). In the cancerous strain the amount of haemosiderin continues to increase until 5 or 6 months after parturition and thereafter shows a very slight decrease, but is still not much below its maximum at almost 11 months. In the non-cancerous strains, on the other hand, there is very little increase after the first month, the amount remaining fairly constant until 5 to 6 months, when it begins to decrease, and by 10 months it has practically disappeared. The history of the haemosiderin in the cancerous strain is not known after 11 months. One uterus only has been obtained which was known to be more than 11 months post-partum and unfortunately the exact period is not known, but in the cleared specimen the dorsal masses of haemosiderin were still clearly visible to the naked eye.

Increase in quantity of haemosiderin is accompanied in both strains by a closer aggregation of the pigment-carrying phagocytes, but in the cancerous strain this aggregation is much more marked than in the non-cancerous strains —the packing is very much tighter. Compare Pl. III, figs. 12 and 11, and 14 and 13, showing uteri of cancerous and non-cancerous strains in pairs of comparable ages. The tightly packed masses in the cancerous strain (Pl. III, figs. 12 and 14) contrast strikingly with the loosely aggregated masses in the non-cancerous strains (Pl. III, figs. 11 and 13). This solid packing of the haemosiderin is as characteristic of the cancerous strain as is the excessive amount of the pigment and its prolonged retention.

There is not the same suggestion of migration of pigment-carrying phago-cytes from the dorsal mass into the mesentery in the cancerous strain as there is in the non-cancerous strain. In the cancerous strain the dorsal mass does not 'tail off' into the mesentery; neither are pigment-carrying phagocytes found within the mesentery (cf. Pl. III, figs. 12 and 11). The occurrence of migration in the non-cancerous strains and its absence in the cancerous strain would account for the closer packing of the haemosiderin in the cancerous strain and for the greater amount, and for its prolonged retention.

scattered clumps of haemosiderin-carrying phagocytes forming dorsal mass

"stream" of pigment carrying phagocytes in mesentery

lumen   **11**

large clump of haemosiderin-containing phagocytes forming dorsal mass

mesentery

lumen   **12**

mesentery

mesenteric wall of uterus

clumps of haemosiderin-containing phagocytes   **13**

large mass of haemosiderin-containing phagocytes
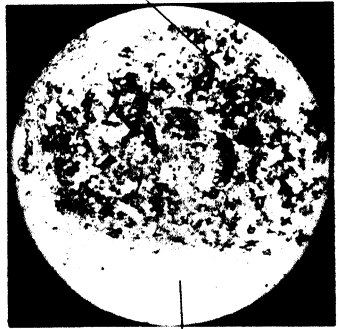
mesenteric wall of uterus   **14**
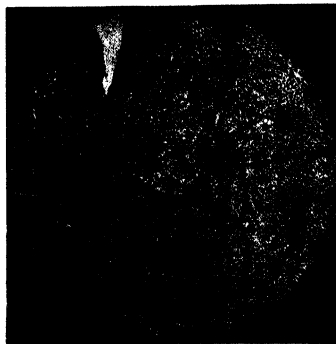
phagocytes containing haemosiderin

mesenteric wall of uterus

15

The whole question of migration requires further investigation and the material available is not what is necessary for the purpose. In most cases the uteri used in this study were dissected out fairly cleanly and only a little mesentery remained attached: no mesenteric lymph glands have been preserved, and the other organs of the body are not available.

CONCLUSIONS AND DISCUSSION

From the foregoing account certain facts emerge clearly. In the first place it is shown that haemosiderin is formed, in all the strains of mice examined, from the blood which is extravasated into the wall of the uterus when the placenta tears away at parturition. In this connexion the most striking facts which emerge, and which are established beyond question, are that in the cancerous strain this haemosiderin is found in much greater quantities than in the non-cancerous strains, and that it is formed somewhat later and persists very much longer. It is by no means certain that these differences are significant with reference to susceptibility to cancer, but they raise some interesting questions and encourage speculation.

In the first place it is not perfectly clear why there is such an excessive amount of haemosiderin in the cancerous strain. Is it that more blood is extravasated into the tissues? Or is it that the breakdown product is disposed of more slowly and so accumulated? The latter alternative seems to be the more probable. There is no evidence whatever of greater haemorrhage at parturition in the cancerous strain, whereas there is abundant evidence of delay in dealing with the extravasated blood. Ingesting of the erythrocytes starts later and goes on for a longer period and the breakdown pigment appears later and persists much longer on the site of formation, as though the phagocytes either fail to carry it away or are extremely slow in the process. It is as though the scavenging is in some way inefficient—as though the phagocytes are either fewer in number or less effective in action. There is no evidence that they are fewer. Histological study shows no scarcity of phagocytes on the scene of action; neither do blood counts suggest any paucity of leucocytes in general, though this latter would, of course, be difficult to demonstrate because of the wide range of variation in normal healthy material. The whole picture, however, suggests slowness or inefficiency of action on the part of the phagocytes rather than paucity in numbers.

An interesting speculation presents itself with regard to susceptibility to cancer. The hypothesis of cellular resistance to cancer put forward by J. B. Murphy (1912, 1913, 1914, 1915, and 1918) and discussed by many later workers, suggests a connexion between the activities of the lymphocytes and susceptibility to cancer. He states that infiltration by lymphocytes accompanies the appearance of a malignant growth. He also says that there is a relationship between the amount of lymphocytic infiltration and the success of a tumour graft—lymphocytosis reducing the susceptibility to tumour transplantation, and lymphocytic leucopenia producing increasing susceptibility. Murphy also found that pieces of spleen introduced into tissue cultures

or implanted in a developing hen's egg interfered with the growth of a tumour.

If the phagocytes, or even the reticulo-endothelial system in general, are indeed responsible for resisting the development and growth of cancerous tissue, and if (as the results of the present investigation suggest) this mechanism exhibits inefficiency in the cancerous strain of mice, this might conceivably be the basis of their susceptibility to cancer.

SUMMARY

1. In mice, haemosiderin is formed in the wall of the uterus from the blood extravasated into the tissues when the placenta breaks away at parturition.

2. The pigment is formed, probably exclusively, within the phagocytes, and forms a mass in the mesenteric wall of the uterus.

3. In mice of the cancerous strain used in this study (R III strain of Dobrovolskaia-Lavadskaia) the haemosiderin is formed more slowly and is disposed of much more slowly than in the non-cancerous strains examined.

4. Phagocytic inefficiency in the cancerous strain is suggested and its possible connexion with susceptibility to cancer is discussed.

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Some Applications of Phase-contrast Microscopy

BY

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With four Plates and three Text-figures

THE purpose of this paper is to present a preliminary report on certain less obvious applications of phase-contrast microscopy which may be of some biological interest. The principles of this method of microscopy are now too well known to necessitate any detailed description (Zernike, 1942; Burch and Stock, 1942).

Briefly, the method sets out to render transparent objects visible by converting changes in phase, which cannot be appreciated by the human eye or by the photographic plate, into changes of amplitude, which can be so appreciated. The theory, as worked out by Zernike (1942), shows that for the case of a grating composed of non-absorbing bars of different refractive indices, the final image is exactly that which would ordinarily be produced by a grating composed of absorbing bars, *provided that the changes in phase produced by the grating are small*. Perhaps the most remarkable feature of Zernike's theory, as applied to a grating, is that it shows the enormous superiority of phase-contrast illumination over other methods for examining transparent material. Without going into details, the image produced by an absorbing grating can be represented by the sum of an infinite number of sine and cosine waves. The image of a transparent grating can be represented by a different series of waves. Ideally we should like to convert this second series into the first, and this is exactly what phase-contrast illumination does! All the other common methods of observation—oblique illumination, central and oblique dark ground illumination, or observation with reduced substage aperture produce images which are represented by different sine and cosine series, which while in some cases showing certain similarities to the 'ideal' series, in general fall far short of it. Roughly speaking, we may say that the effect of phase contrast illumination is as if we stained the object with a dye which stained each point with an intensity proportional to the product of its thickness and refractive index. To this extent, and subject to the performance of the microscope, phase-contrast illumination gives a true representation of what is actually present. All other methods commonly employed to render transparent objects visible produce an image which is often a mere caricature of the object.

It must be pointed out that this almost exact theory has only been worked out for very special objects, such as gratings, the elements of which produce

very small changes of phase. It is reasonable to ask how small must these changes be, and what happens if they become large. It is not easy to give an exact answer to these questions since the mathematics involved is rather complicated. Fortunately, we can tackle the whole question in a much more general way, and without assuming any particular type of object.

Following Zernike's treatment the object (or the image) can be represented by a vector diagram as in Text-fig. 1a.

Each point is represented in phase and amptitude by a vector. Areas which absorb light without affecting its phase will be represented by vectors lying

1a

1b
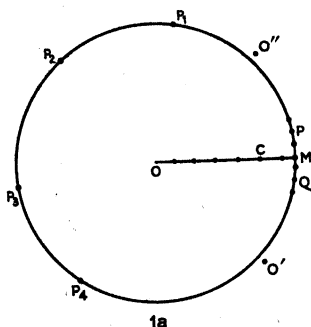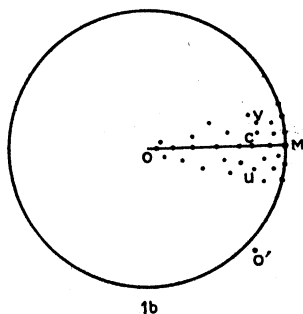
TEXT-FIG. 1a.   TEXT-FIG. 1b.

along the line OM. Areas which absorb no light but merely alter its phase (i.e. transparent objects) will be represented by points on the circumference of a circle. Now according to the Abbe theory of the microscope, the final image is produced by interference between the central undiffracted image of the object and a series of diffraction images. In order to obtain phase contrast it is necessary to change the phase of the central pencil relative to the phases of the diffracted pencils. In practice this is usually accomplished by inserting a phase-retarding or phase-advancing strip into the back focal plane of the objective, and an illuminating diaphragm of corresponding shape in the sub-stage. The phase strip is usually constructed to produce a 90° change of phase in the central image. The reason for this is that the exact theory for the case of a grating requires such a phase change in order to transform a 'phase-grating' into an 'amplitude grating'. In practice, however, and in dealing with a more general type of object, this phase change need not be exactly 90°. Indeed in some cases it is advantageous to produce a phase change other than 90°.

Returning to our vector diagram (Text-fig. 1a) the effect of the central pencil alone can be represented by a vector which is the average of all the vectors representing the object. Let us suppose it to be represented by the line OC. The effect of the phase strip is to rotate this vector through 90°, or in other words to shift the origin of the system from O to O' (positive phase contrast) or O'' (negative phase contrast). We can now see that non-absorbing

points, which are represented by vectors such as P and Q on the circumference of the circle, will appear to have very different intensities when the origin is shifted to O' or O''. Whereas with normal illumination the intensities of such points were represented by $(OP)^2$ and $(OQ)^2$, i.e. equal and therefore showing no contrast, with phase-contrast illumination the intensities become $(O'P)^2$ and $(O'Q)^2$ or $(O''P)^2$ and $(O''Q)^2$. Restricting ourselves to positive phase contrast (i.e. origin at O') we see that highly refracting points will appear darker, less refracting points lighter, than the mean illumination.

Now suppose the object contains many points which produce large changes in phase. The effect of such points will be represented by vectors OP_1, OP_2, OP_3, OP_4, &c., around the circumference of the circle. If these points are sufficiently numerous the point representing the average of the whole system will approximate to O. Thus phase-contrast illumination would be of no value for such an object. At the same time we observe that the intensity of a point producing a phase change θ will be the same as the intensity for points producing phase changes of $2n\pi + \theta$, i.e. an extremely refractile region may not always appear any darker than a less refractile region.

OBSERVATIONS ON LIVING INTACT ANIMALS

In practice, according to Zernike (1942), phase-contrast illumination is most useful for objects which produce phase changes of less than $45°$ (one-eighth of a wave-length) since objects thicker than this can be seen reasonably well by other methods. In most cases this means that phase contrast is best used with very thin objects. Single cells such as protozoa or tissue cultures are ideally suited for this method of examination. The excellent films produced by Michel (on the development of grasshopper spermatocytes) and by Hughes (on the growth of cells in tissue cultures) have already shown the enormous potentialities of the method for this type of material. Unfortunately, the insistence on the use of *thin* objects has rather tended to obscure the point that it is not thickness *per se* that is important, but the amount of phase change. Phase-contrast illumination may be quite useful for examining relatively thick but very transparent objects. Now the larva of *Chaoborus* (the phantom larva) has long been known for its transparency, and it was thought that it would provide good material for examination by phase-contrast illumination. The larva is usually several millimetres in diameter but can be flattened somewhat by mounting in water under a cover slip. At the same time this immobilizes the insect, but if carried out gently does not appear to harm it, for the latter will swim away quite normally when released after examination. Now it must be admitted that many of the cells and tissues of *Chaoborus* can be observed by the familiar method of reducing the substage iris to a very small diameter and altering the focus. As a matter of fact this is one method (though a very poor one) of producing phase contrast. Resolving power is lost owing to the small substage aperture, the method only works properly when the object is slightly out of focus, diffraction effects are accentuated, and the image is a very poor representation of what is actually present. With

properly adjusted phase-contrast illumination the results are very different. It is possible to state with reasonable confidence that we can now observe living cells in the living intact multicellular organism with greater clarity, and with a greater chance of seeing what is actually present, than ever before. Pl. I, fig. 1, shows a low-power general view of part of a living *Chaoborus* larva, focused on two ganglia with the intervening nerve-cord. Pl. I, figs. 2 and 3, are higher power views of single ganglia, taken with the ⅛ in. objective. These photographs show one large nerve-cell on each side of the ganglion. The nuclei and nucleoli are clearly seen and it will be observed that the cytoplasm is highly granular. These granules are particularly well seen in Pl. I, fig. 3. It is not yet possible to establish their nature with certainty. At first sight they might be taken for Nissl granules, and indeed their appearance in Pl. I, figs. 2 and 3, closely resembles that in fixed sections stained with Borrel's methylene blue, a recognized Nissl stain. It is possible, however, that the granules seen in the living cells may be part of the Golgi apparatus, which is often very easily seen in other living cells by phase-contrast illumination. Another region of some interest is shown in Pl. I, fig. 4. It is necessary to speak with caution of such material as the tissues of living insect larvae, but as a result of prolonged observations I am of the opinion that Pl. I, fig. 4, is a photograph of a living motor nerve-ending in a muscle-fibre. (All the muscles of *Chaoborus* larvae are composed of single fibres.) The pyramidal accumulation of sarcoplasm containing one or more nuclei with what appears to be a nerve-fibre entering at the apex corresponds to the description of the 'Doyères hillock' given in the entomological literature (see Morison, 1927). Pl. II, fig. 5, shows another view of these end-plates taken at the same magnification but from a smaller specimen. Two end-plates are seen (they lay on slightly different planes and a compromise focus had to be chosen in order to show both on the same photograph) and they appear to be interconnected by two nerve-fibres which seem to emerge from a common junction, at which there appears to be some sort of cell, possibly a peripheral nerve-cell, but more probably a neurilemmal cell. Numerous sarcoplasmic granules are also seen. Other insect larvae are also suitable for study by this method, e.g. young mosquito and *Chironomus* larvae (Pl. II, fig. 6). The giant salivary gland chromosomes in the intact *Chironomus* larva can often be seen quite well. Numerous possibilities exist for the study of secretory and excretory activity of cells in insect larvae. It is very much to be hoped that suitable vertebrate material may be found. I have carried out preliminary observations on the tissues of a tadpole's tail and the results were quite promising. Unfortunately, these observations were made rather late in the spring when frog tadpoles were very scarce and rather large. The transparent tails of very young tadpoles should be quite suitable. The mesenteries of small vertebrates have not on the whole been found satisfactory, though very clear views of the capillary endothelium have sometimes been obtained. It is possible that a very thin transparent chamber inserted in the ear of a rabbit may prove satisfactory.
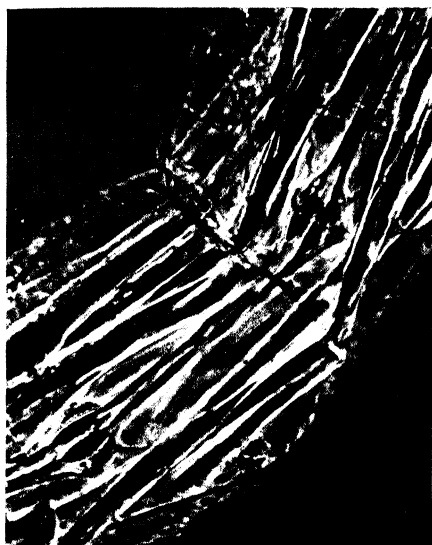
Fig. 1

Fig. 2

Fig. 3

Fig. 4

FIG. 5

FIG. 6

FIG. 7

FIG. 8

R. BARER.---PLATE II
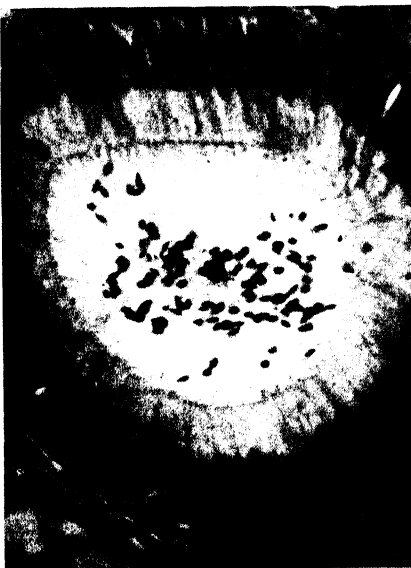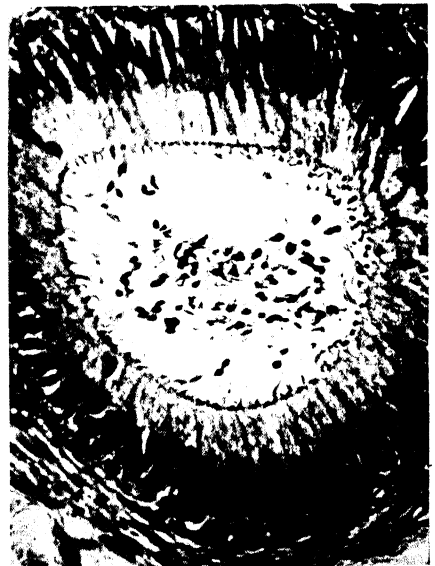
THE EXAMINATION OF STAINED SECTIONS

It is generally stated that phase-contrast illumination is of no value for examining stained objects. This opinion seems to have been based mainly on theoretical consideration of an over-simplified ideal case. In Text-fig. 1 we saw that Zernike represented all absorbing points as lying on the line OM, and all phase-changing points as lying on the circumference of the circle. When the origin is shifted from O to O', the absorbing points on OM become roughly equidistant from O' and therefore lose contrast. Now a stained section will contain points like U and V (Text-fig. 1*b*) which both absorb and produce changes of phase. The intensities of such points will be proportioned to $(O'U)^2$ and $(O'V)^2$ and they will appear to have good contrast, whereas with ordinary illumination (origin at O) they will appear equally stained. We thus have the theoretical possibility that phase-contrast illumination may after all be of some value for examining stained objects. To put the matter very crudely we should expect to lose much of the contrast due to the staining, but we might gain a different contrast due to differences in refractive index in both the object and the stains. (In a private communication Dr. Burch has suggested that stains may have anomalous refractive indices in the region of their absorption bands, and that it would be interesting to examine stained sections by phase-contrast illumination using monochromatic light of different wave-lengths, in order to vary staining contrast.) It must be admitted at once that this simple theory is far from adequate. Unfortunately, the exact treatment bristles with difficulties, and, indeed, it is unlikely that the problem will ever be solved for any but very special cases. In the circumstances it was judged best to try the method in practice. A wide range of routine normal and pathological material was examined, but a systematic examination of different stains and mounting media has not yet been made.

There are several ways in which phase-contrast illumination may be of value for examining stained sections. Perhaps its most obvious use is to render visible details of structure which have not been properly stained by the dyes employed. Such cases occur especially in certain histochemical reactions. Text-fig. 2*a* shows a section of bone marrow stained by the Feulgen technique. Only the nuclei are visible. Text-fig. 2*b* shows the identical field as seen by phase-contrast illumination. The cytoplasm as well as the nuclei of the cells is now rendered visible.

Other examples of the way in which unstained or poorly stained details can be made clearer are shown in Pls. II and III, figs. 7–10. It should be pointed out that it is often extremely difficult to demonstrate the superiority of phase-contrast illumination over ordinary illumination by means of photographs. The reason for this is that one automatically tries to get the best possible photograph out of any given material. Details which are seen with difficulty through the microscope can be rendered clearer by filters, differences in exposure, development, and printing. Strictly speaking one ought to treat both phase-contrast and ordinary pictures identically, but this is very difficult

as the former require longer exposures. A direct comparison under the microscope is far more convincing than any series of photographs. Were it not for a natural tendency to 'load the dice' against oneself the photographic superiority of phase-contrast would be even more clear-cut.

Neurological material offers a particularly interesting field for study by means of phase-contrast microscopy. It is well known that scarcely any two methods of staining nervous tissues will produce the same result. As regards
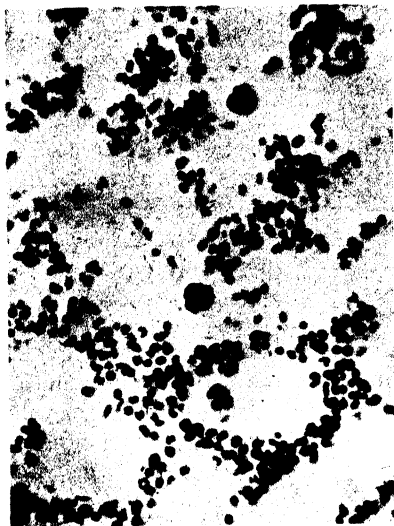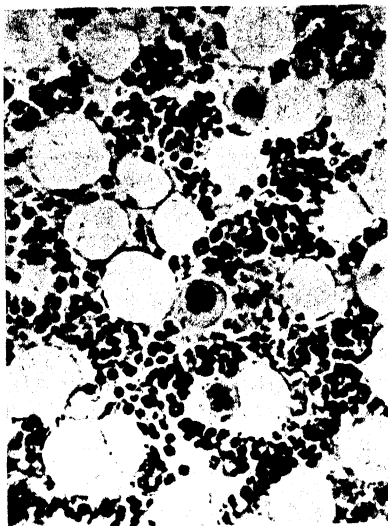
TEXT-FIG. 2a.   TEXT-FIG. 2b.

the various methods of silver staining, one may almost say that the same method in the hands of two different workers will produce different results. It is, therefore, of some interest to know what an entirely unstained nerve-cell looks like. The answer is seen in Pl. III, figs. 11, 12.

These cells were almost invisible by ordinary illumination, even when the substage iris was almost closed, and were quite impossible to photograph, except by phase-contrast illumination. The appearance is more or less what one might expect to see if it were possible to use a mixture of silver staining and Nissl staining. It is the purpose of this paper to describe the possible applications of a new technique rather than to discuss results, but for the present we may say that the nerve-cell, free from staining artifact (but not, of course, free from fixation artifact), may show appearances suggestive of Nissl granules, Golgi apparatus, *boutons terminaux*, and numerous extremely fine cell processes. A further investigation on frozen-dried material and tissue cultures is being undertaken.

Returning, however, to the question of stained nervous tissue, in general
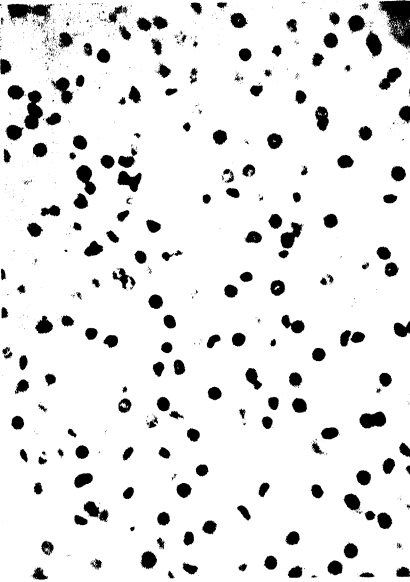
FIG. 9
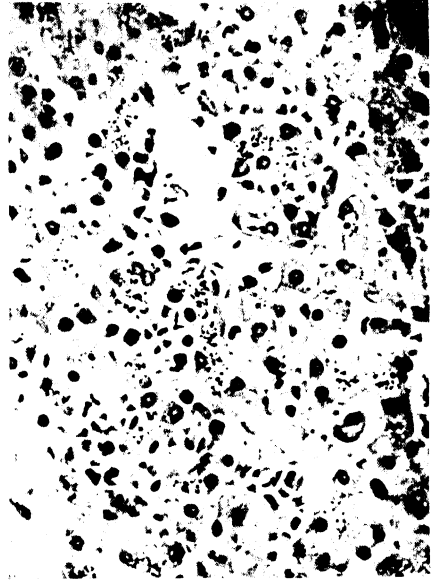
FIG. 10

FIG. 11

FIG. 12

FIG. 13

FIG. 14
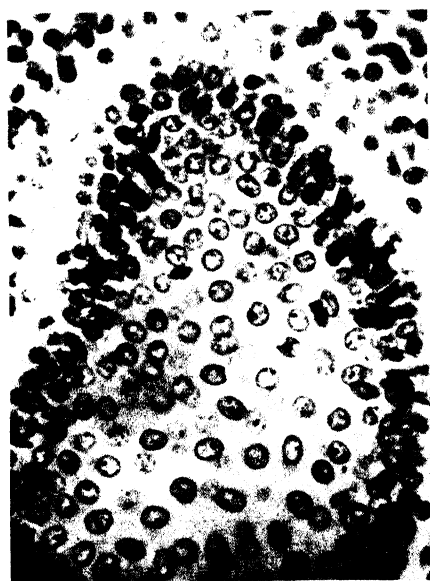
FIG. 15
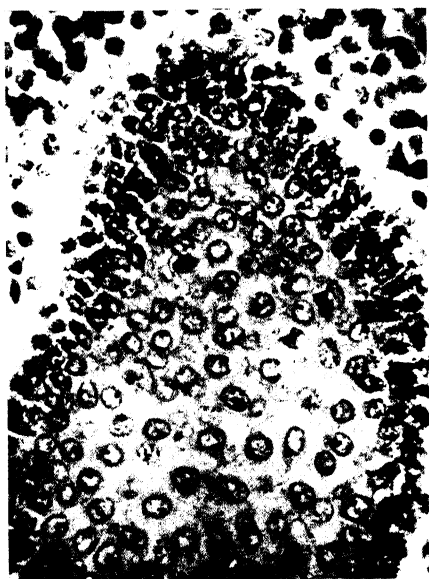
FIG. 16

we may say that any given stain is fairly specific for either nerve-cells or nerve -processes. Thus toluidin blue, methylene blue, and cresyl violet stain nuclei and Nissl granules but not nerve-fibres, whereas the reverse is on the whole true of most silver stains. We thus have the possibility of rendering the missing element visible by phase-contrast illumination.

Pl. IV, fig. 13, shows the Purkinje cells of the cerebellum seen in a Bielschowsky preparation. The nerve-fibres are very clear but the cells seem almost devoid of detail. Pl. IV, fig. 14, shows the same field by phase-contrast
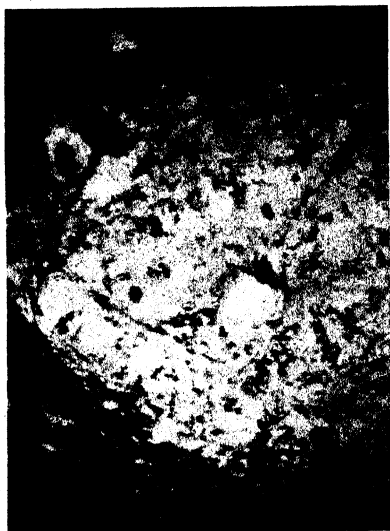
TEXT-FIG. 3a.

TEXT-FIG. 3b.

illumination. The nuclear structure is clearly visible. Conversely it is often possible to render the nerve-fibres more visible in Nissl preparations by means of phase-contrast. Finally, it is necessary to mention the various myelin stains, used for studying nerve-tracts. These usually leave the nerve-cells unstained and the latter can be rendered visible by phase-contrast illumination. Text-figs. 3a, 3b show this effect in a Weigert-Pal preparation (×150) of a transverse section of the spinal cord (monkey).

The main disadvantage of the method is that it very often renders too much detail visible. Most stains are to a certain extent selective, but phase-contrast illumination may reveal so many minute fibrils that it is very easy to miss the wood for the trees. A considerable amount of further study will be necessary before any exact evaluation of the method as applied to neurological material can be made.

Many types of granules are extremely easily seen by means of phase-contrast illumination. Pl. III, figs. 9, 10, shows a section of liver from a case of pernicious anaemia. Iron-containing pigment granules (haemosiderin) are

present but very difficult to see by ordinary methods. Phase contrast reveals the presence of myriads of these granules with remarkable clarity. Even such normally well-seen pigment granules as melanin are rendered clearer by phase contrast. Pl. IV, figs. 15, 16, from a section of a melanoma of the skin illustrate this point. The method has also been used with success for demonstrating secretory granules. It should be mentioned that all the photographs of fixed material shown were from routine paraffin sections, 8–12 μ thick, mounted in Canada balsam.

DISCUSSION AND CONCLUSIONS

It is necessary to emphasize once more that it is not the purpose of this paper to discuss results in detail, but rather to suggest certain applications of phase-contrast microscopy which require further study. As regards the examination of living cells and tissues anyone who has ever seen such material by properly adjusted phase-contrast illumination would readily acknowledge its superiority over the older methods. The main problem here is to find suitable material. Isolated cells such as bacteria, protozoa, tissue cultures, or spermatozoa are of course ideal, but multicellular organisms are in many ways more interesting and important. Insect larvae and other transparent creatures such as certain medusae may yield useful information, but it would be very valuable to be able to extend the range of available material, especially to vertebrate tissues. The examination of stained sections by phase-contrast illumination is still in its infancy. Time and further investigation alone will show its uses and limitations. All that can be said at present is that it may prove to be a useful adjunct to the usual methods for examination of certain types of material. It is not possible to predict what will be seen by its aid. In some cases certain details may be rendered more clearly visible, in others the method will be found of no value. One practical point should be emphasized: the appearance of a stained section under the low power ($\frac{2}{3}$ in. objective) is often bizarre and disappointing. The investigator should not be disturbed by this but should always proceed to examine the section with the $\frac{1}{6}$ in. objective, when the appearance is often greatly improved. I am at present unable to offer any satisfactory explanation for this phenomenon, but recent experiments suggest that appearances can be greatly improved by proper choice of colour filters. One last word: it is of course very important that any new method of microscopy should be received with caution. It must be admitted that there are many theoretical and practical points about phase-contrast illumination that require much further study. Nevertheless the theoretical basis underlying the method is sufficiently well understood to enable one to say that the image seen by its use bears at least as close a relation to what is actually present as that seen by more orthodox methods. Indeed, it is necessary to point out that the theory underlying the observation of stained objects by ordinary illumination is very far from complete. The theory underlying the use of oblique or central dark ground illumination is even less perfectly understood, but this has not prevented the extensive use of these

methods. The proper course to pursue for the present is to use the method cautiously as an adjunct to other routine methods and to seek out its special applications and limitations.

I wish to thank Professor W. E. Le Gros Clark, F.R.S., for his encouragement and advice. I have had several valuable discussions on histological matters with Drs. A. Brodal, P. Glees, and G. Bourne. Mr. A. W. Dent and Mr. L. G. Cooper have provided technical assistance. Finally, I wish to express my special gratitude to Dr. C. R. Burch, F.R.S., for many stimulating discussions on all aspects of phase-contrast microscopy.

SUMMARY

Some extensions of the simple theory of phase-contrast microscopy are considered. It is emphasized that *transparency*, rather than *thickness*, is the limiting factor for the successful employment of the method. Certain transparent insect larvae (*Chaoborus, Chironomus*) can be observed in the living state by phase-contrast illumination.

The statement that the method is of no value for the examination of fixed and stained sections is based on consideration of an ideal physical case. In practice the method may be a valuable adjunct to routine examination of such material. Examples are given of the application of phase-contrast microscopy to normal and pathological stained sections.

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EXPLANATION OF PLATES

All the figures are untouched photomicrographs taken with the Cooke, Troughton, and Simms phase-contrast equipment. Green filter. Orthochromatic film.

PLATE I

Fig. 1. Low power view (phase contrast × 150) of two segments of a living *Chaoborus* larva. Focused at the level of the nerve-cord and ganglia. Two large nerve-cells are visible in the lower ganglion.

Fig. 2. Higher power view (× 500) of a single ganglion showing two nerve-cell nuclei and nucleoli.

Fig. 3. Another example (× 500) showing the granules in the cytoplasm of the large nerve-cell on the right.

Fig. 4. Motor nerve-ending (phase contrast × 500) in striated muscle-fibre of a *Chaoborus* larva. Note the nucleus.

PLATE II

Fig. 5. Another example of motor nerve-endings; same magnification as Fig. 4 (× 500) but from a smaller specimen. The two muscle-fibres lay on slightly different levels and a compromise focus was chosen. Note the sarcoplasmic granules and the apparent inter-connexion of the nerve-fibres.

Fig. 6. Nucleus and nucleolus of a cell in an anal appendage of a living *Chironomus* larva. Phase contrast × 500.

Fig. 7. Epididymis tubule. Haematoxylin and eosin. Normal illumination. × 500.

Fig. 8. Same field. Phase contrast. The details of the poorly stained ciliated epithelium are now clearly seen.

PLATE III

Fig. 9. Liver in pernicious anaemia. Haematoxylin and eosin. Normal illumination. × 500.

Fig. 10. Same field. Phase contrast. The tiny haemosiderin granules are made visible.

Fig. 11. Anterior horn cells of spinal cord of a monkey. Unstained. Alcohol-acetic acid fixation. Phase-contrast. × 500.

Fig. 12. Anterior horn cell of monkey. Unstained alcohol-acetic acid fixation. Phase contrast. × 1,200.

PLATE IV

Fig. 13. Purkinje cells, cerebellum. Bielschowsky's silver impregnation. Normal illumination. × 500.

Fig. 14. Same field. Phase contrast. Note nuclear detail and great increase in number of fibrils.

Fig. 15. Melanoma of skin. Haematoxylin and eosin. Normal illumination. × 500.

Fig. 16. Same field. Phase contrast. Melanin granules become more sharply defined. Note also the intercellular bridges.

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TO THE

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PREPARED BY

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PREFACE

THE arrangement of this Index of Volumes 62–87, inclusive, follows closely that of the two previous ones, namely, Volumes 1–28 and 29–61.

Authors and subjects are in the same alphabetical series—the former printed in capital letters. If an author has more than one paper they are numbered chronologically in brackets; the title of papers by two or more authors is given in full for the first-named author only.

Where there is more than one author with the same surname, direct references are given to all except the author with the longest series of papers, which are treated in the usual way.

Numbers referring to volumes are printed in heavy type, those referring to pages in ordinary type.

<div style="text-align: right">H. P. G.</div>

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